

THE POSTNATAL GROWTH IN BODY WEIGHT OF THE CAT

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ONE FIGURE

There seem to be but very few papers dealing with the growth of the cat either before or after birth. One of us has studied the prenatal growth of the cat (Latimer, '31 a and '31 b). Hoesslin ('26) gives the growth of four kittens beginning at five weeks of age, but only one of these was on a normal diet. There are several papers describing the early embryology. The junior author collected the data on the growth of twelve kittens and the senior author is responsible for the preparation of the paper. Although the number of cases will not warrant definite conclusions nor statistical treatment, yet the paucity of published material on the growth of the cat seems to justify this brief report.

This report is based on the weights of six female and six male kittens from five litters born in March, May, and June, 1917. Each kitten was weighed at birth and every day for the first few days and then at frequent intervals thereafter. Two specimens, the two females in litter 7, were weighed at birth, eight hours after birth, and at twenty-four hours of age. The kittens in all but one litter were weighed for thirteen weeks. This one litter was weighed up to the seventy-fourth day, and the age of the last weighings of the other litters varied from ninety-one to 152 days. All of the kittens were left with the mother cats until they were weaned. After weaning they were fed table scraps, milk, and live mice from

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the laboratory stock. They always had access to this food as soon as they could eat anything solid.

All of the weighings plotted on age in days are shown in figure 1. The curve drawn through the averages for each week for the first eight weeks is practically the same for both sexes. After this time the males grow more rapidly than the females.

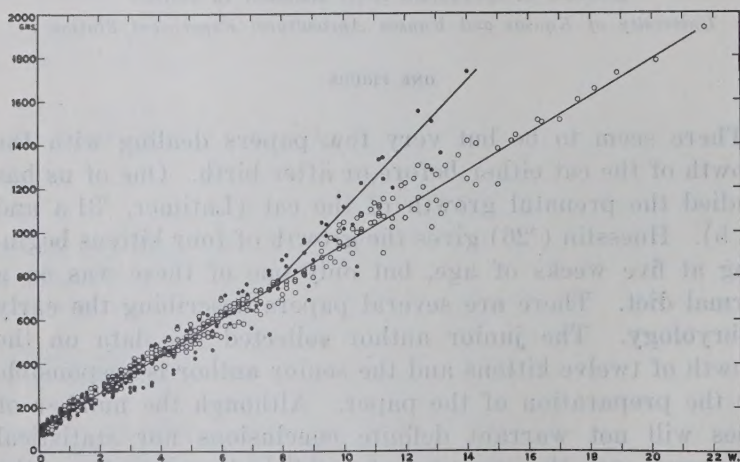


Fig. 1 The individual weights in grams for all specimens are shown plotted on age in weeks. The circles represent the females and the dots, the males. The lines are drawn through the averages for each week for each sex. During the first three weeks the cases were so close together that many had to be omitted.

Table 1 gives the average birth weight in grams for males and females and the average weight for each week for each sex, together with the range of individual weights.² It is interesting to see that the average weight of the females is heavier at birth and for the first five weeks, after which the males are slightly heavier for two weeks and then decidedly heavier. These averages were obtained by getting the average weight of each specimen for the week and then getting the average of these average weights. The range of weights is for the individual weighings.

² The complete data will be filed with The Wistar Institute.

Both the curves in figure 1 and the data in table 1 show no postnatal decrease in weight. The individual daily weights of ten of these kittens for the first three weeks show no weighing less than that of the preceding day. Two specimens, a male and a female, both from litter no. 9, show a slight decrease in weight on the third day. The average gain for the males for the first twenty-four hours after birth is 11.86 grams, ranging from 9.4 to 19.8 grams. The similar average for the females is 13.58, ranging from 5.6 to 23.00

TABLE 1

The range and the average body weights in grams at birth and for each week

AGE IN WEEKS	SIX FEMALES		SIX MALES	
	Range	Average	Range	Average
Birth	97.0-119.5	103.92	82.7-107.4	97.75
1	97.0-212.2	144.21	82.7-195.9	129.22
2	162.4-295.8	230.22	145.6-282.3	213.10
3	266.5-377.1	323.68	259.0-365.0	296.52
4	330.2-475.4	402.42	266.4-486.7	363.73
5	387.2-562.7	467.02	346.0-577.8	446.03
6	466.7-522.6	540.30	420.0-624.5	541.01
7	520.5-701.4	622.30	514.7-766.8	641.69
8	644.8-760.0	683.80	558.7-820.4	713.92
9	715.3-817.1	762.53	589.0-963.0	810.98
10	790.0-1032.0	891.35	872.4-1159.0	1005.58
11	897.3-1104.7	1008.60	789.0-1218.9	997.90
12	902.0-1216.0	1010.65	1200.0-1347.0	1280.25
13	1024.0-1361.0	1202.10	1271.0-1550.0	1440.33

grams. The average increase for the second twenty-four-hour period is 14.86 for the males and 12.68 grams for the females. This average does not include the two negative increments of 5.1 and 11.5 grams for a male and a female, respectively. The average gain for the first week for the males was 87.32 grams and for the females, 90.4 grams. Two females were weighed eight hours after birth in addition to the weighings at twenty-four hours of age. One of these gained 1.7 grams and the other, 7.2 grams, or gains of 1.75 per cent and 6.03 per cent, respectively. The first cat gained 14.9 grams between the eighth and the twenty-fourth

hours and the second cat gained but 7.3 grams in this same period. Many more animals would be necessary to establish this lack of a postnatal decrease in the cat, but these few weights are interesting because of the lack of this decrease immediately after birth and because of the uniform growth in the females for the first thirteen weeks.

The data in table 1 give the averages for each sex, but, in studying the material, it was seen that there was a difference in the average weights of the different litters, and so a table was prepared giving the average weight at birth and for each week for each litter. To save room, this table is not given, but it shows that the lighter newborns are, in general, lighter for the first thirteen weeks of postnatal life. Four of these five litters with average birth weights ranging from 88 to 105 grams were sired by the same male and these four litters contain the extreme variation in weight at birth and at the thirteenth week. The mothers were all different. The adult male weighed 3580 grams and the mother cats, shortly before pregnancy, weighed from 200 to 1000 grams less. The litter having the heaviest average weight at thirteen weeks was from the heaviest female, which weighed 3370 grams at the beginning of pregnancy.

It is interesting to note that at the thirteenth week the curve for the male kittens has reached a little over 40 per cent of the weight of the adult male. The female average is 320 grams less.

Kaufman ('30), comparing the postnatal growth of the chick and the pigeon, finds that the chick loses weight at first, while the pigeon increases rapidly in weight after hatching. In so far as these meager data permit, we may conclude that the cat, unlike man, has no period of postnatal decrease. Both sexes grow at about the same rate until the eighth week, after which the rate of growth of the males is greater.

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ATTEMPTS TO REDUCE THE GILLS IN NEOTENOUS NEWTS, *TRITURUS VIRIDESCENS*

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THREE PLATES (TWENTY FIGURES)

Animals are neotenuous that retain their larval form much longer than is usual for their group. There are two kinds of neoteny in amphibians, the partial type of certain anurans in which metamorphosis occurs late, but before sexual maturity, and the total neoteny of a few urodeles that become sexually mature while they still have some or all of their larval structures. Urodeles are characteristically slow in metamorphosing, but among them there are different degrees of neoteny. These include such forms as *Cryptobranchus* and *Necturus*, whose gills have thus far resisted experimental attack, the axolotl larva of *Ambystoma tigrinum*, and a number of species cited by Kollman ('82) (*Triton vulgaris*, *T. cristatus*, *T. alpestris*, *T. boscai*, *T. waltli*) in which all individuals have tendencies toward neoteny or else occasional ones are neotenuous. Recently, Noble ('29) reported neoteny in the newts, *Triturus viridescens*, collected at Woods Hole, Massachusetts. In one gilled specimen also having the other larval characteristics he found the testis cysts packed with sperm, thus showing that "some specimens of *T. viridescens* may reach sexual maturity while still larvae" (Noble, '29, p. 7). This neotenuous individual was exceptional. But among the newts collected from the same ponds Noble found many sexually mature ones that had partially taken on adult form (i.e., had lost body fin and Leydig cells from the skin and had molted), but still retained their gills. Partially meta-

morphosed newts like these were the subjects of experiment in the present study.

The object of these experiments was to reduce the gills, thus completing metamorphosis, and to find out whether the neoteny was a type influenced by environmental factors or was more or less insensitive to them. If the latter were true, it would be evidence indicating that the hereditary background of these newts differed from individuals of *T. viridescens* that lose their gills early and have a red or land phase. It would point toward the possible genetic origin of neoteny in this newt as suggested by Noble ('29). The gill reduction was attempted by means of, 1) forced residence in a dry environment, 2) transplants of anterior-pituitary lobe from gill-less to gilled newts, and, 3) transplants of thyroid gland from gill-less to gilled newts.

HISTORY

Neoteny has been reported in several European salamanders, such as those already quoted from Kollman, *Triturus taeniatus* (Van Swinderen, '25), and *Salamandra maculosa* (Germershausen, '10). In the Mexican race of *Ambystoma tigrinum*, often mentioned in European literature, the axolotl is persistently neotenuous. In the western race of the same species, found in the Rocky Mountains and New Mexico, it is sensitive to environmental changes and frequently metamorphoses upon being removed from its native habitat (Swingle, '22 b).¹ Many experiments have been made in attempting to find the causes of neoteny. They have been mostly concerned with varying the environmental factors, and with the administration of endocrine glands or their products.

In 1876, Chauvin reported that the young Mexican axolotl could be made to metamorphose by forcing it to grow gradually accustomed to terrestrial life. This became increasingly difficult when the animals were older. Much later, Huxley and Hogben ('22) compared the time required for thyroid-

¹For this and other references not included in the bibliography, see article by B. M. Allen ('29).

induced metamorphosis in these axolotls with that necessary for them when they were forced to live on land. They found the latter method slower, and that it took longer for sexually mature than for younger animals. In 1923, Hogben repeated some of Chauvin's experiments and reported that metamorphosis did not generally occur in larvae of six to nine months that were kept in shallow water from which they could emerge easily. Evidently, axolotls will not leave the water unless they are forced to do so. Investigations show that temperature is a factor in neoteny; cold is an agent in the neoteny of *Salamandra maculosa* (Germershausen, '10) and in *Triturus alpestris* (Wichand, '06). The forced metamorphosis in axolotls (Huxley and Hogben, '22) was hastened by an increase in temperature.

In his experiments on feeding thyroid to tadpoles Guder-natsch ('12-'14) showed the importance of internal secretions in anuran metamorphosis. Since then an extensive study has been made of the rôle of the thyroid and pituitary glands in this process in both urodeles and anurans of various ages.² Especially pertinent to this paper are experiments on neotenuous anurans, *Rana clamitans* and *R. catesbiana*, adult *Necturus*, and axolotls. Thyroid feeding (Swingle, '18 b, '22 b, '23 a) and thyroid transplants from adult anurans and axolotls are capable of inducing metamorphosis in neotenuous anurans, but thyroid administered to *Necturus* and axolotls is only effective in the latter (Swingle, '22 a, '23 a; Smith, '23). Iodine will also metamorphose both neotenuous anurans and the Colorado axolotl even if thyroidectomized or hypophysectomized (Ingram, '29 a; Swingle, '23 b; also see Allen, '29). Considerable work has been done on administration of anterior pituitary by grafts or injections. Swingle ('21, '22 b) and Ingram ('29 b) have metamorphosed *Rana catesbiana* and *R. clamitans* with pars-anterior transplants, and Hogben ('23) and Spaul ('24, '25), the Mexican form with injections of anterior-lobe extracts.³ This work as well

² See review of literature by Allen ('29).

³ Smith, however ('23, '25), has found anterior-hypophyseal substance to have a retarding influence on spontaneous or thyroid-induced metamorphosis in the Colorado axolotl.

as much of that on hastening metamorphosis in non-neotenus forms has led to the histological demonstration of the stimulating action which such anterior-pituitary administration exercises on the thyroid (Uhlenhuth and Schwartzbach, '28; Ingram, '29 b, '30; Grant, '30, '31). In general, these investigations show that metamorphosis is closely associated with the combined normal functioning of these two glands.

This brief survey indicates that in amphibians and especially in urodeles, neoteny has been modified by forcing the animals to live on land, by varying the temperature, and by treatment with endocrines. The present experiments on *Triturus viridescens* apply some of these same tests, forced residence on land, and administrations of pituitary and thyroid gland.

MATERIAL

Three types of newts (*T. viridescens*) were used in the experiments, gilled and gill-less ones from Woods Hole and gill-less adults from South Hadley, Massachusetts. Most of the Woods Hole newts examined (over 500) were collected in October and November, 1930; about forty were captured in the following March and April, 1931. They varied between 6.2 and 9.6 cm. in length and from 0.93 to 2.9 grams in weight. Although of adult or nearly adult size, 75 per cent of them had gills, but the presence or absence of gills could not be correlated with the size of the animals—a condition noted by Noble ('29, p. 7). The South Hadley newts (300) were collected in October and November, 1930, also. They varied from 7.4 to 9.7 cm. in length and were more uniform in size than the newts from Woods Hole. None of these nor hundreds of similar ones showed a trace of gill rudiment.

The gilled newts from Woods Hole uniformly bore three gills on each side (fig. 5) in which circulating blood could be seen when they were examined with a binocular microscope. In forty newts the average length of the gills was 0.13 cm., but their size did not correspond with that of the animal. One newt measuring 9.6 cm. bore gills (fig. 5); another of equal length had none. Some individuals but 6.2 cm. long

had completely lost their gills, while others of the same length bore conspicuous ones (fig. 3). In nearly all cases the branchial clefts were closed; in one newt 8.6 cm. long, they were open.

The coloration of the newts was fairly typical of the species, with characteristic variations ranging from olive-black or olive-brown to light olive-green. Eighty-five per cent of all the gilled newts were darkly colored and the entire Woods Hole group was generally darker than the South Hadley one. The black spots were usually larger in the Woods Hole animals than in the South Hadley adults, particularly on the ventral side (fig. 1), but the vermilion spots of the dorsal side were noticeably smaller in the former (figs. 16, 17). The skin of the South Hadley newts was also rougher and more papillated than that of the Woods Hole ones (figs. 17, 16). Sections of the skins of animals from both places revealed no fundamental difference in structures (figs. 13, 14, 15, 18, 19, 20). No Leydig cells were found in the epithelium of any of the types (gilled or gill-less). The epidermis of the South Hadley newts was slightly thicker and definitely more folded than that of the Woods Hole ones, and its skin glands were much larger.

The condition of the secondary sex characters and the sex organs in the Woods Hole newts varied from partial to complete maturity. Toe pads and roughened areas on the hind limbs were slightly developed in 50 per cent of the males when they were collected. In some males the bases of the tail fins were 0.3 cm. wider than those of the females. The usual measurements were 0.53 cm. for the male and 0.41 cm. for the female. The cloacae of the males were large and of the females characteristically smaller. Sections of the testes of two gilled males killed on October 17, 1930, contained spermatozoa, as shown in the photograph (fig. 9). In the ovaries the majority of the eggs were small; some scattered ones approached full size, this seeming to be independent of the size of the animal. The oviducts were slender and the fat bodies small, even difficult to find by gross exami-

nation (fig. 7). The gonads of the gill-less Woods Hole newts were similar to those of the gilled ones. The secondary sex characters, sex organs, and fat bodies of both sexes of the South Hadley newts were typical of the adult development of *T. viridescens* in autumn. In fifteen individuals examined the fat bodies were conspicuous—a contrast to their small size in the Woods Hole newts (fig. 7).

Microscopic examination⁴ of the thyroids of six gilled (Woods Hole) newts suggested that these glands were in a state of low activity. The follicles were frequently oval and were surrounded by flattened epithelium. The cell boundaries were not clearly defined, cytoplasm was scanty, and the flattened nuclei lying parallel to the surface of the follicle seemed nearly in contact with each other. The colloid contained in the follicles was homogeneous. The character of this gland corresponds to thyroids of low activity that Uhlenhuth ('27) found in neotenus and larval salamanders. The thyroids of six gill-less Woods Hole newts suggested a less marked degree of inactivity. Though many of the follicles were oval, some were rounded. In the follicular epithelium there were both flattened and cuboidal cells. The cytoplasm was more abundant and the nuclei were often rounded. Though the colloid contained in the follicles was usually homogeneous, in one case chromophobe vacuoles were numerous (compare Uhlenhuth, '27).

While the appearance of the thyroid glands in adult South Hadley newts may vary considerably, those examined showed marked activity. The spherical follicles were surrounded by cuboidal epithelium composed of clearly defined cells with abundant cytoplasm. Chromophobe vacuoles were numerous at the border of the colloid. These glands appeared to be of a type described by Uhlenhuth ('27 b) in adult *Ambystoma opacum*.

⁴ A more detailed study of the thyroids in this form is under way.

EXPERIMENTS TO INDUCE METAMORPHOSIS IN GILLED NEWTS,
T. VIRIDESCENS*Forced residence in a dry environment*

These experiments were made in an attempt to hasten the reduction of gills in the newts collected at Woods Hole. On October 14th, two lots of twenty gilled newts were separated into four groups each, according to length and weight. Both lots had well and uniformly developed gills averaging 0.13 cm. in length. The first lot of four groups was kept in glass jars cushioned with earth and moss. This was occasionally sprinkled sparingly with water, but it was never allowed to accumulate. The second lot was kept in jars of water. A third lot of ten typical aquatic adults (collected in South Hadley) was subjected to land residence and a fourth set of ten aquatic adults was kept in water. All of the animals were kept in the same room, at an average temperature of 57°F., and given the same rations. Those living in the water ate readily, but the others usually refused food and it was necessary to feed them forcibly. Both lots of animals were kept over six months in these conditions.

The gilled newts behaved very differently when kept from their water habitat. They crowded under the moss and huddled together there, showing no sign of life except the rhythmic quivers of pharyngeal respiration. If disturbed, they sought a new hiding place. Twice each week at feeding times, they were placed in shallow dishes of water for not longer than five minutes, so that their gills might be examined.

Of the twenty newts subjected to the dry conditions on October 14, 1930, ten lived for seven months or more. At the beginning of the experiment these had gills 0.05 to 0.1 cm. long. At the end of the seven months or more land residence, their gills had become dry and shrunken (fig. 2), but when moistened, these soon expanded and circulating blood was distinctly visible within them. One newt kept on land fifteen weeks (fig. 6) was placed in water for five hours. Within this time its gills expanded (fig. 4) and were the same size as those of its control that had been living an equal length of

time in water. Sections of the gill of a similar fifteen-weeks animal showed an abundance of pigment (fig. 12) as compared with one kept in the water (fig. 11).

The skin of the land dwellers became glazed and smooth, like drying frog skin (fig. 6), but did not in any way appear adapted to exposure to air. They became a little paler than those in the water (fig. 2). So far as could be ascertained, molting occurred as frequently in land as in the water habitat.

The tail fins became smaller in the land residents of both sexes of the gilled newts. But their entire bodies were shrunken and there was evidently little or no compensatory growth, and what was true of the gilled animals in these respects held also for typical aquatic adults (South Hadley) subjected to land residence as further controls. The skin of the latter, however, became rough and papillated.

Homoplastic transplants of the anterior lobe of the pituitary gland from typical gill-less adults to the gilled newts

The accelerative effect of anterior-pituitary substance upon metamorphosis is well known and its application was naturally included in these attempts at gill reduction.

The donors selected were typical aquatic adults collected in South Hadley during October and November. The recipients were twenty-one gilled newts (nine males, twelve females) collected at Woods Hole in October and November. These were given biweekly grafts of anterior pituitary from the donor adults for nine weeks (November 13, 1930, to January 13, 1931). One pars anterior was transplanted each time to the dorsal shoulder muscles of the newts. Two groups of gilled newts were kept as controls. One set of these (twenty-one newts) received grafts of pars intermedia and nervosa of the pituitary, and the others (twelve) were kept in water under the same conditions as the experimental ones, but without treatment.

After the third transplant of anterior pituitary, the animals became slimy and most of them molted. After this, molting

occurred more often in these than in the controls. This skin reaction was followed by a gradual enlargement of tail fins in both sexes. In the males the average tail width (at the base) increased from 0.46 to 0.71 cm. after five transplants carried over eighteen days. In tails that were more than 0.7 cm. wide, the transparent fin was rippled along the dorsal border. In the females the average tail width increased from 0.42 to 0.61 cm. after six transplants and a small transparent fin appeared on every one. In addition to the enlarged tail fins, the males developed black toe pads and roughened areas on the hind limbs typical of the breeding season (figs. 10a, 10b).

Following the sixth transplant, twenty days after the beginning of the experiment, four females began to lay eggs, and continued to deposit one to eight eggs at frequent intervals. No developing embryos were ever found. Examination of the gonads of five animals (three females and two males) that died after thirteen and fourteen transplants showed that the ovaries contained more full-size eggs than those of control animals killed at the same time (fig. 8). In egg-laying animals examined, the oviducts were correspondingly larger than in the controls. Motile spermatozoa in the cloacae of the males and their total absence in the cloacae of the controls indicated that the testes had been stimulated by the transplants. No spermatophores were discovered.

Microscopic study of the thyroids of six experimental animals suggested that these glands had been stimulated by the anterior-pituitary substance. Both rounded and oval follicles were present, and a few resembled follicles which had released their colloid. The epithelial cells ranged from the low to the high cuboidal type, with cytoplasm plainly evident. The nuclei were oval and not closely contiguous. In rare instances, small chromophobe vacuoles could be seen in the colloid of the follicles.

The gills of the experimental animals showed no noticeable change in size and appearance either while the grafting was in progress or while the newts were under observation during several weeks afterward.

There were some changes to be noted in the two sets of control animals carried with this experiment. In the group that received transplants of pars intermedia and nervosa of the pituitary there was some darkening of the skin. In all of the controls the secondary sex characters, gonads, and thyroid glands appeared typical of untreated, gilled newts. No measurable change occurred in the gills.

Ten of the unoperated control newts were living on May 11, 1931, and still retained their gills. There was, however, some indication that a very slow reduction of them was taking place normally. On November 10, 1930, the average length of gills in ten unoperated newts was 0.12 cm.; on May 11, 1931, it was 0.07 cm. This slight reduction suggests that these newts might lose their gills if given sufficient time.

Transplants of thyroid gland from typical gill-less adults to gilled newts

In view of the part that the thyroid is known to play in amphibian metamorphosis, transplants of these glands were also made. The donors of the thyroids were typical gill-less adult newts (South Hadley) that were also the donors of pituitary glands in the preceding experiments (p. 14). The recipients were twenty gilled newts (Woods Hole) averaging 7.46 cm. long, with gills averaging 0.13 cm. long. Two thyroid glands were transplanted biweekly into their dorsal muscle. The transplanting began on December 3rd. The controls for this experiment were the group receiving implants of pars intermedia and nervosa (p. 14).

The experimental animals molted soon after the first transplant and continued to do so about as often as the newts that received the transplants of anterior pituitary. No effect of the transplants was apparent upon secondary sex characters or gonads. After eight transplants, not only the tail fins but the entire bodies of both sexes became shrunken. The animals grew thinner in spite of the fact that they ate eagerly. It was impossible to discover any reduction of the gills during the time that the animals survived, ten to twenty-seven days following the first transplant of thyroid tissue.

DISCUSSION

When collected, about three-quarters of the *T. viridescens* from Woods Hole had gills; the remainder did not. Outside of this, there was no noticeable difference between them in size, external appearance, or in development of gonads. Since gilled and gill-less newts of the same size were found in the same places, their gilled condition does not appear to be dependent on differences in food or in age. It should be noted here, however, that in the laboratory a very slow decrease in the size of gills took place in certain individuals among the gilled newts kept in water for a long period. And, there was some indication that this occurred more frequently in the better-nourished, larger newts and hence may be slightly correlated with size.

It seemed possible that changing the environment or varying the available amounts of pituitary or thyroid secretion might affect the gills and thus indicate that the neoteny was due to an external factor such as environment or to an internal one (endocrine secretion). If such experiments did not affect them, the presence of the gills could then be attributed to a genetic cause more deep-seated than mere failure to complete the full genetic expression. Other than from this viewpoint, no study of the neotenuous condition from a genetic angle was attempted. Van Swinderen's ('25) experiment with *Triturus taeniatus* might well have been tried on these newts had fertile gilled ones been available. He bred a metamorphosed male (*T. taeniatus*) with a neotenuous female which produced thirty young, half of which proved to be neotenuous.

The gills of *T. viridescens* persisted after the newts had lived in a dry place throughout the winter and spring (p. 13). During these seasons, their reaction to dryness was either too gradual to be discovered or else there was none at all. This indicated that the neoteny was deep-seated in these newts, and that their persistent gills were expressive of the long-extended larval condition common among their urodele relatives. They appeared to be outcroppings of old stock whose pattern was not easily erasable.

The results of the dry-environment experiments made here are different from those obtained by Noble ('29) on gilled *T. viridescens* collected from the same ponds at Woods Hole. The retention of gills made it seem probable to him that the newts did not go through any land stage. He tested this supposition by placing, on July 11th, "seven recently metamorphosed newts with prominent gill-rudiments" (Noble, '29, p. 9) in a jar with damp moss, but no free water. By August 8th (twenty-eight days later), all traces of gill rudiments had been lost from the terrestrial animals, while in the control series kept in water, the gills showed no sign of reduction. Two tests were made during July and August of the same year with like results. It is possible that in midsummer these neotenus newts are susceptible to changes which do not affect them earlier. It may be that the gilled stage is then naturally reaching its end. This has not been demonstrated by controls under observation, but it is possible that their development has been retarded by laboratory residence. In 182 days (November 10th to May 11th) the average length of the gills of ten controls, kept in water, decreased nearly half (0.12 cm. to 0.07 cm.), the decrease becoming noticeable only in the spring. But this decrease was very slow and had not been nearly completed even a month later. Among similar controls, dating from October 24th, most of the individuals had well-defined gills in the following late July. In the experiments recorded in this paper, gilled newts forced to live on land for eight months showed no effect of their change of environment.

Low temperature has been thought to play an important rôle in the neoteny of salamander and triton larvae (Huxley and Hogben, '22) and *Triturus vulgaris* larvae (Boettger and Schwarz, '28). In the present instance, temperature cannot be effective, since some of the *T. viridescens* kept at 70°F. showed no more tendency to lose their gills than those kept at 57°F.

Marked development of secondary sex characters and stimulation of gonads followed transplants of anterior pituitary

into gilled newts. There appeared to be a limited thyroid reaction. The gills were unaffected. Reactions of the sex apparatus and the thyroid gland have been recorded in several amphibians (p. 9). After transplants of adult pituitary, young *Ambystoma* larvae developed secondary sex characters before metamorphosis and their gonads were stimulated (Burns, '30). By implants of hypophysis into urodele embryos, Blount ('30) caused a tendency toward precocious sexual development, although the larvae metamorphosed with the controls. Egg laying was induced in adult *T. viridescens* by Adams ('30) and in *Eurycea bislineata* and *Desmognathus fusca* by Noble and Richards ('30) and Noble ('30).

That transplants of anterior pituitary stimulate the thyroid has been shown by Uhlenhuth and Schwartzbach ('26 and '28) in *Ambystoma* larvae, by Grant ('30, '31) for larvae of *Necturus*, *Ambystoma jeffersonianum*, and *A. opacum*, and by Ingram ('29 b, '30) for *Rana clamitans*. In these cases the follicular epithelium changed from a squamous, or low cuboidal, to a high cuboidal or columnar type, colloid was lost from the follicles, an increase in the amount of chromophobe colloid occurred, and chromophobe vacuoles appeared within the cells—expressions of increased activity of the gland. The Golgi apparatus also suggested increased activity (Ingram, '30). In the urodeles (except *Necturus*) and anuran larvae, such changes were accompanied by metamorphosis including loss of gills. While some indication of thyroid stimulation occurred after the anterior-pituitary transplants in *Triturus viridescens*, they apparently did not stimulate the thyroid sufficiently to bring about gill reduction. Nor did thyroid grafts, a method of supplying the thyroid hormone directly, induce any change.

The above-described lack of reaction on the part of the gills of *T. viridescens* to both pituitary and thyroid administration is not wholly surprising. It is generally conceded that the anuran metamorphoses more readily than the urodele in response to thyroid gland and its substances. This has

has been shown to be true in *Ambystoma* by Uhlenhuth ('22 b). Nevertheless, *Ambystoma* and *Spelerpes maculata* can be made to metamorphose by thyroid feeding, thyroid transplants, iodine administration, or anterior-lobe transplants (Uhlenhuth, '22 b; Swingle, '22 b, '23 a; Uhlenhuth and Schwartzbach, '28; Uhlenhuth and Winter, '29; Huxley and Hogben, '22; Grant, '31). While axolotls do respond to thyroid feeding and inorganic iodine (Swingle, '22 b; Ingram, '29 a; see also Allen, '29), some difficulty has been encountered with the response of the axolotl to transplants of their own thyroids (Hogben, '23; Swingle, '24) and to administration of anterior-pituitary substance (Smith and Smith, '22; Smith, '23, '25). To some extent the failure of the pituitary transplants to cause gill reduction in *T. viridescens* seems similar to the retarded metamorphosis reported by Smith ('25) in the Colorado axolotl after administration of anterior-pituitary substance, and the marked tail-fin growth also suggests that recorded by him. He ('31) is inclined to believe that the latter is a growth response due to a hormone of the anterior lobe which does not stimulate the thyroid, but possibly depresses its activity. While these experiments give some slight support to the activation of the thyroids as judged by the histological examination of a few, this response was not marked. However, the point to be emphasized is that the gills remain insensitive not only to pituitary treatment, but also to considerable amounts of thyroid substance, although the egg laying and molting after the former and the molting and emaciation after the latter indicate that the implants were potent in certain ways.

SUMMARY

1. In *Triturus viridescens* reduction of gills was not induced by forced residence in a dry environment for seven months or more (beginning October 14th) of winter and early spring. At the end of these experiments blood was circulating in the gills. Land residence reduced the size of the body in gilled and gill-less newts (collected at Woods Hole) and in typical

aquatic adults (collected at South Hadley). Although given forced rations, they probably took less food than they would have done in their normal habitat.

2. Reduction of gills could not be induced by homoplastic transplants of anterior lobe of the pituitary or of thyroid gland.

3. After homoplastic transplants of the anterior pituitary, the gilled newts laid eggs in December and January and motile spermatozoa were present in the cloacae of the males. Tail fins and pads on the toes and legs of the males also developed after this treatment.

4. After receiving thyroid grafts, gilled newts became very thin and molted frequently.

5. In sections of material taken in February the thyroid glands of both gilled and gill-less *T. viridescens* from Woods Hole appeared to be less active than the thyroids of adult *T. viridescens* from South Hadley. There was a slight indication that the thyroids of the gilled newts were less active than the gill-less ones taken at Woods Hole.

6. An extremely slow decrease in size of gills was evident in some individuals kept in water. In so far as this is true, there is an indication that nourishment and size may be correlated with gill reduction. But presence of gills has not been found to correlate with size in freshly collected animals.

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PLATE 1

EXPLANATION OF FIGURES

1 Ventral sides of *Triturus viridescens* ($\times 2$). Left, gilled form from Woods Hole with large spots; right, gill-less form from South Hadley with smaller ones.

2 *T. viridescens*, gilled form ($\times 2$). Left, animal kept in a dry environment for seven months, October 14, 1930, to May 15, 1931, showing shrunken pigmented gills, generally lighter color and smaller size than control (right) that was kept in water.

3 *T. viridescens*, gilled form (natural size).

4 *T. viridescens*, gilled form (slightly less than natural size); same animal as in figure 6, but photographed after being in water five hours. Note position of gills and dullness of skin.

5 *T. viridescens*, gilled form (natural size); control animal kept in water. Note gills.

6 *T. viridescens*, gilled form (natural size); kept in a dry environment from October 14, 1930, to January 28, 1931, and photographed while dry, showing gills and shiny skin.

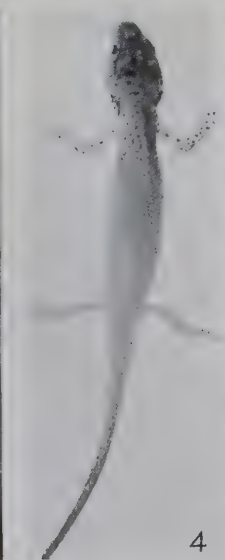


PLATE 2

EXPLANATION OF FIGURES

7 Left, gill-less form from South Hadley, showing the prominent fat body (*fb*), ovaries (*ov*), and oviducts (*od*); right, gilled form from Woods Hole with inconspicuous fat body and small ovaries and oviducts. $\times 2$.

8 Gilled newts, Woods Hole ($\times 2$). Left, control animal killed October 14th; center, control killed December 24th; right, animal killed December 24th, after receiving thirteen homoplastic transplants of anterior pituitary. *od*, oviduct; *ov*, ovary.

9 Section of testis ($\times 215$) of gilled newt, killed October 11th, showing spermatozoa.

10 a and b Gilled newts, Woods Hole ($\times 2$); *a*) control animal; *b*) newt, showing development of leg and toe pads after eleven homoplastic transplants of anterior pituitary.

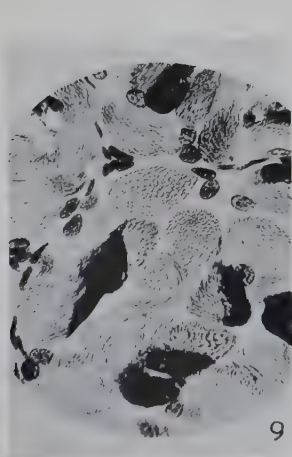
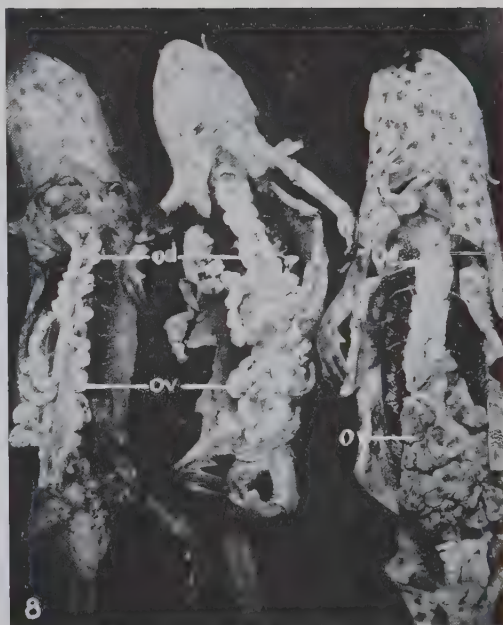
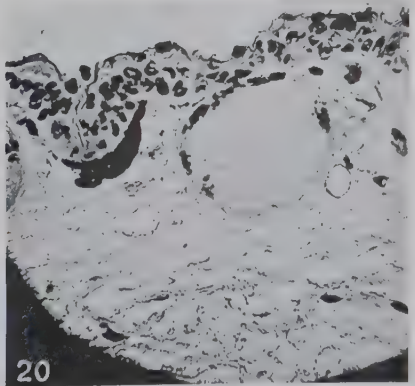
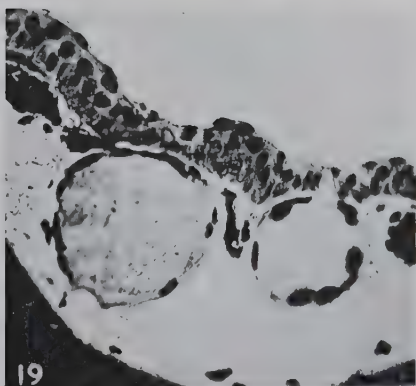
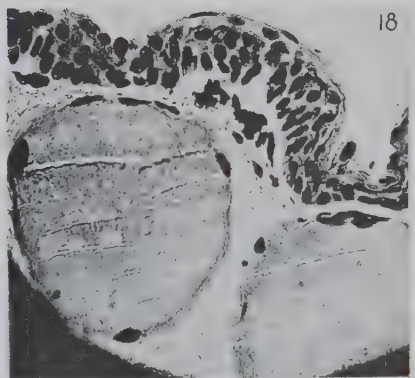
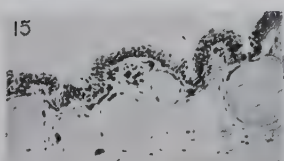
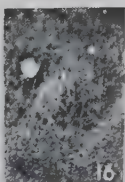
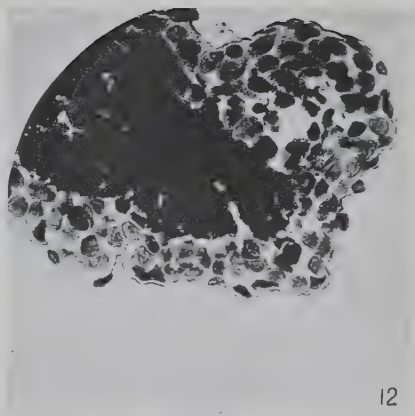
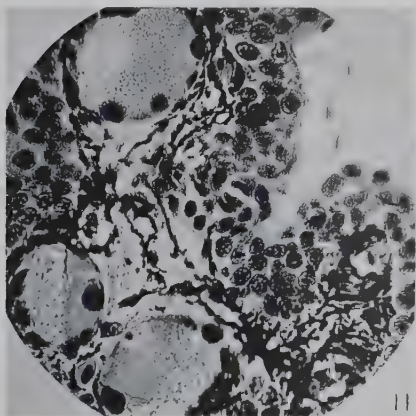


PLATE 3

EXPLANATION OF FIGURES

Triturus viridescens.

- 11 Transverse section of gill of newt kept in water. $\times 215$.
- 12 Transverse section of gill of newt kept in dry environment for fifteen weeks. $\times 215$.
- 13 Section of skin from side of the body of gilled newt, Woods Hole. $\times 100$.
- 14 Section of skin from side of the body of gill-less newt, Woods Hole. The epithelium is smooth, the skin glands small, and the chromatophores often expanded. $\times 100$.
- 15 Section of skin from side of the body of gill-less newt, South Hadley ($\times 100$). Note the thicker, folded epithelium, contracted chromatophores, and large skin glands.
- 16 Surface view of skin on side of the body of gilled newt, Woods Hole, kept in a dry environment from October 14th to January 28th. $\times 4$.
- 17 Surface view of skin on side of the body of gill-less newt, South Hadley, kept in same conditions as above. Figures 16 and 17 were made on the same negative. $\times 4$.
- 18 Region from skin shown in figure 15, gill-less newt, South Hadley. $\times 215$.
- 19 Region from skin shown in figure 16, gilled newt, Woods Hole. $\times 215$.
- 20 Region from skin shown in figure 14, gill-less newt, Woods Hole. $\times 215$.



THE INTERACTION BETWEEN THE OMENTUM OF THE RABBIT AND TRANSPLANTED ORGAN PRIMORDIA OF THE SAME SPECIES

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ONE PLATE (FIVE FIGURES)

INTRODUCTION

Isolated mammalian tissues have been grown in tissue culture and in grafts with considerable success, showing that they are capable of growth and differentiation provided they are cultured in a suitable medium. With this in mind, an effort was made to find some region of the adult rabbit to which isolated embryonic primordia could be transplanted. The capacity for independent self-differentiation exhibited by such transplants constitutes a measure of the determination of 'embryonic segregation' (Lillie) which has taken place in the primordium up to the time of isolation. Transplants were made to the loose areolar connective tissue between skin and body wall, to the peritoneum lining the body cavity, to the mesenteries, and to the omentum. Little if any differentiation was secured from any of these regions except the omentum. Transplants inserted in the omental bursae of young and adult rabbits were found to be immediately incorporated and vascularized by proliferating host capillaries. In this environment the primordia exhibit a capacity for independent self-differentiation which is comparable to control embryos of the same temporal age.

It is not the purpose at the present time to describe the results of these experiments, as they will appear in a subsequent paper, but to note the method used in the experiments

and to analyze the processes by which the transplant is incorporated, vascularized by proliferating omental blood blood vessels, and eventually removed. The grafts were not allowed to remain on the omentum a sufficient length of time to show the complete story of the removal of the graft, but there is evidence that this latter phenomenon also occurs in a very specific manner.

It might be expected, from the peculiar types of tissue which go to make up the omentum, that such a degree of stimulation would be produced by the transplant that it would stand little chance of survival once the defense reaction of the connective tissue was initiated. Instead, the omental tissues not only seem to tolerate the presence of this embryonic fragment for a relatively long time, but the immediately adjacent tissues quickly grow around the transplant and become fused with those of the primordium. The omentum incorporates the transplant and its blood vessels proliferate and invade the embryonic tissues, thus insuring the necessary food supply and elimination of waste materials. In this sense the reaction of the omentum is quite similar to that of the chorio-allantoic membrane of the developing chick toward transplants of chick primordia.

Several workers have reported observations upon the phenomenon of incorporation in chorio-allantoic grafts. Danchakoff ('18), in studies of the grafts of adult spleen, finds that the constituent parts of the allantois respond in a specific manner to the presence of the transplant. In addition to a local thickening of the immediately adjacent tissues, the ectodermal cells of the chorio-allantoic membrane which are in contact with the transplant undergo stratification and proliferation to form numerous papillae which penetrate the graft tissue. Through a partial destruction of the capillary net of the allantois, hemorrhages are produced which, together with the ameboid activity of the free cells of the graft (lymphocytes, macrophages, etc.), are instrumental in the loosening and disintegration of the ectodermal layer. By this means the transplant sinks within the mesenchyme of the

allantois and is invaded by host capillaries. Granular leucocytes are not conspicuous in either graft or host tissue at early stages, while the erythrocytes remain inert and are ingested by phagocytic cells. Danchakoff concludes that the principal effect of splenic grafts is exerted on the host mesenchyme, while the response of the ecto- and entodermal layers is local and secondary, due to mechanical stimulation.

Minoura ('21) finds no special reaction of the ecto- and entodermal layers to transplants of testis and ovary. The grafts are completely incorporated and usually show an abnormal accumulation of leucocytes in the host tissue. Vascularization is established as early as twenty-four hours after the operation. Sections of grafts reveal four definite zones: first, the mesoderm between the ectodermal and entodermal layers in which the graft lies; secondly, a vascular area encircling the graft; thirdly, the growing region of the graft; and fourthly, an inner necrotic area of variable extent depending upon the rapidity with which vascularization occurs (compare Willier, '24). In some cases the central area may be liquefied and absorbed by phagocytes.

Willier ('24) mentions the presence of leucocytes in grafts and the effect of the delay in vascularization, in that those areas farthest from the blood supply become necrotic. The ectoderm undergoes abnormally numerous mitoses and may proliferate so as to enclose partially the graft.

Huxley and Murray ('24) state further that the host tissue in the vicinity of the graft thickens extensively. In addition, the ectoderm shows evidence of stratification, cornification, and of marked hyperplasia, while only occasionally does the entoderm undergo hyperplasia to form several layers. More recently Henderson ('30) has made a further study of this process. She finds that the transplant first becomes fused to the chorionic epithelium and a definite thickening of this layer together with that of the mesenchyme ensues. As the graft sinks into the mesenchyme the ectoderm at the margin of the transplant grows over it and finally, at about forty-eight hours, completely encloses the transplant. During this

process the underlying chorionic epithelium breaks, and through the opening thus made host mesenchymal cells and blood vessels grow into the graft, with the result that the differentiating parts of the transplant not only become surrounded, but also separated by host tissue. After thirty-one hours of incubation vascularization is well begun. Previous to this time the transplant has apparently lived either upon nutrient material contained within its cells or upon nourishment absorbed indirectly from host vessels through the intervening ectodermal layer.

Certain of the details of the process of incorporation into the omentum are quite similar to those observed in chorio-allantoic grafts, while others are quite different. In omental grafts (compare Danchakoff, '18, and Hoadley, '24) there is a large amount of extravasated blood present throughout the tissue spaces of the graft and filling all organ cavities. This is so characteristic of omental grafts that it will be dealt with in detail.

I wish to thank Prof. A. B. Dawson for his many helpful suggestions and criticisms given during the course of this investigation.

MATERIAL AND METHOD

Isolated primordia of ten- to twelve-day rabbit embryos were transplanted to the omental bursae of young rabbits, of adult males, and of pregnant and non-pregnant females. This was accomplished by partially withdrawing the great omentum through an incision in the left side of the rabbit just posterior and lateral to the stomach and spreading the omentum upon filter-paper previously moistened with warm Ringer-Locke solution to prevent sticking. Frequent applications of this solution were made from time to time to minimize drying and to prevent chilling of the omental tissues. The withdrawal of a major portion of the omentum is necessary in order that transplantation may be made to a region in which fat accumulation is at a minimum. A small opening

is made in the ascending or ventral wall of the sac in a region relatively free from large blood vessels and fat. It is not always possible to avoid the smaller blood vessels, so that frequently small capillary hemorrhages occur. If bleeding into the omental bursa is excessive the transplant fails to adhere to the mesothelium.

The primordia together with a small amount of Ringer-Locke solution are then squirted by means of a warm pipette into the omental cavity, several similar or dissimilar primordia being introduced into the same omentum. Care must be taken not to introduce any considerable amount of fluid with the primordia, else they fail to stick to the mesothelium, slip into that portion of the cavity around the stomach, and are lost. After the primordia are introduced, the omentum is returned to the abdominal cavity and the incision closed. Sterile instruments and fluids were used.

It is very difficult to control the final position of the transplants after they have been introduced into the sac by the pipette. If not too sticky (e.g., limb buds) they may be moved to a limited extent by gently rubbing the outer surface of the omental wall with a smooth, blunt object. Their position depends chiefly on chance. It is possible that a small transplant that came to rest in the vicinity of a good histiocytic aggregation, or one that became lodged in a region relatively free from blood vessels, would stand a poorer chance of survival. These factors, together with the injury received at the time of operation and the various pressures and tensions to which the transplant is subjected as it differentiates, may account for the variability and malformation so conspicuous in many cases.

There is no difficulty in distinguishing the larger grafts from the omental tissues even when considerable fat surrounds them. In addition to being large, well-rounded bodies (fig. 1), many of them are deep red in color from extravasated blood. A graft cleared in cedar oil and examined microscopically shows relatively large vessels leading to and from it, and these in turn are continuous with the main blood-

distributing channels (fig. 2). On the other hand, some grafts may be so small, colorless, and surrounded by fat, e.g., isolated ear grafts, as to be detectable only by gently rolling the tissue between the fingers. The presence of cartilage in such grafts furnishes the resistance by which the graft is detected.

For the present study, grafts were removed at three, five, seven, nine, eleven, thirteen, and fifteen days following transplantation. To facilitate the study of specific cellular reactions, such as those of the histiocytes, some of the animals were injected three to four times preceding the operation with warm lithium-carmin solution in Ringer-Locke, according to the method of Maximow. By this means the histiocytes (macrophages) were marked to ensure their identification. The grafts were fixed in Helly's modification of Zenker's fluid and were stained in hematoxylin azur-eosin.

Various modifications of this technique have been employed for the purpose of testing the effects of the operation and of the anesthetic not only upon the embryos in utero, but also upon the grafts. Furthermore, males have been used as hosts to see if the sex of the host might play some rôle in graft incorporation and differentiation. It may be said at this point that neither the anesthetic, age, nor the sex of the host has any noticeable effect upon either the process of incorporation of the transplant or the degree of differentiation attained by the constituent parts of the primordium. Males or females may be used as hosts with the expectation that identical results will be secured.

The does used in these experiments were secured as desired from Prof. W. E. Castle, of Bussey Institution, Harvard University. They represented cross-bred material used in Professor Castle's genetic studies. All matings were made with one active buck which had been raised from a preceding stock.

OBSERVATIONS

A. The omentum and its reaction to the graft

The omentum of the rabbit consists of a double fold of the mesogaster (portion of the dorsal mesentery) which forms

a large collapsed sac, the omental bursa, just dorsal and caudal to the stomach. One edge of this sac is bounded by the elongated spleen. The cavity contains a small amount of liquid, the serous exudate, just sufficient to moisten the walls of the sac which are in contact, but free to slide over one another. The tissues of this serous membrane include a thin layer of peculiarly modified, loose, irregularly arranged, connective tissue covered by a layer of mesothelium. All of the cellular elements characteristic of diffuse, loose connective tissue are found in this membrane, but they are more numerous. In addition to an abundant vascularization and fat accumulation along the edges of the sac, the omentum is especially well provided with fibroblasts, pericytes (undifferentiated mesothelial cells), lymphocytes, plasma cells, mast cells, and eosinophilic leucocytes. In the so-called milky spots (*tâches laiteuses*) and along the larger blood vessels, the histiocytes are aggregated in large numbers; elsewhere many of them are differentiated as spherical ameboid macrophages which ingest dye in animals vitally stained with lithium carmine.

Under various pathological and experimental conditions the omentum has been shown to take an important part in the defense reaction against local injuries. The presence of irritating foreign materials in the peritoneum intensifies the activity of the cellular components of the omentum and the number of cells present, especially macrophages, increases greatly. The introduction of a primordium within the omental bursa might be expected to cause a reaction sufficient to remove completely the embryonic material. Instead, the omental tissues tolerate the transplant for a relatively long time. It is true that the transplant is quite large, and if only partial incorporation took place it would take a comparatively long time for its complete removal, inasmuch as individual cells (chiefly histiocytes) constitute the destructive and absorptive agents. In the meantime considerable differentiation might have taken place.

Usually the transplant is completely incorporated between the third and fifth days (fig. 3). In some instances the incorporating overgrowth may be quite thick, forming a broad band of omental tissues around the differentiating transplant, while in others this band may be but a few cells in thickness. In a few cases no encapsulating layer was found, and only a narrow strip of tissue, but a few cells in width, is present on each side of the transplant. Incorporation has been delayed, but the degree of differentiation attained by the constituent parts of these transplant is comparable.

Differentiation and incorporation go on side by side. In fact, incorporation is not a limited process, but progresses throughout the life of the graft. The encapsulating band of omental tissue increases in thickness and blood vessels continue to proliferate, eventually reaching all parts of the growing graft. The graft gradually increases in size, and if two or more developing grafts are in close proximity they usually form one large compound body. This last occurs in later stages of growth and accounts for the difficulty of estimating the number of original transplants which have 'taken'.

Incorporation is usually performed by one wall of the omental sac, although in a few cases both walls have been seen to be involved. The first step in this process is the adherence of the transplant to the mesothelium. This localized stimulation subsequently results in an aggregation of cellular elements to form a definite thickening of the omental connective tissue in the vicinity of the transplant (Danchakoff, '18; Huxley and Murray, '24). Conspicuous in the thickened area are fibroblasts, macrophages, granular leucocytes, and other connective-tissue elements, together with a number of proliferating capillaries and lymphatics (fig. 3).

The next step in the process of incorporation involves the breakdown of the intermediate mesothelial layer, allowing the transplant to come into intimate association with the connective tissue. The transplant sinks into the thickened area of the omental wall, possibly through pressure exerted by such environmental agencies as the proximity of the opposite

wall of the omental sac and contact of other organs of the abdominal cavity. There is no evidence of fusion having occurred between mesothelium and ectoderm of the transplant preliminary to the breakdown of the former. The mesothelial layer is normally only one cell thick and shows no mitotic activity following transplantation such as has been shown to occur in the ectoderm of the serosa of the chorio-allantoic membrane in response to the presence of a transplant. In some manner, possibly through the activity of wandering cells, the mesothelial layer undergoes dissociation and its cells either mingle with those of the transplant or are destroyed. At the same time fibroblastic aggregations of host tissue exhibit a conspicuous orientation toward the transplant. Consequently these also are probably instrumental in the dissociation of the mesothelial layer. Following this preliminary step by which the transplant comes into contact with the connective tissue of the omentum, capillaries invade the tissue of the transplant, while marginally the connective tissue together with the mesothelium proceeds to grow around the transplant.

As nearly as can be determined, proliferating capillaries are the only constituents of the host tissue to invade the transplant to any extent. Host blood cells soon appear in the tissue spaces of the graft, but these are introduced by way of the blood stream, being forced by arterial pressure through the delicate endothelial walls of the invading capillaries. This will be discussed in the section dealing with the presence of extravasated blood throughout the graft tissue spaces. Henderson reports not only an invasion of blood vessels, but also host mesenchyme in chorio-allantoic grafts. The results of the present experiments seem to show that the differentiated elements of the host mesenchyme may invade the periphery of the graft, especially in those types which become completely embedded in host tissue, but the encapsulation of parts within the graft by marked fibroblastic aggregation appears to be the result of the differentiation of graft mesenchyme. The fibroblastic aggregations which encapsulate the

graft are of host origin. In older compound grafts, host tissue derived from the primary encapsulations may be seen between the several original units.

Grafts are commonly of two types. In one type where very little superficial ectoderm was included with the transplant, the union between host and donor tissues is practically complete. The epithelium present has been isolated and appears as cavities and cysts in the walls of which mitotic figures are abundant, especially in the germinal layer. This epithelium continues to differentiate to form the various layers of the epidermis. In some cases (e.g., limb-bud grafts) hair papillae are conspicuous (compare Hoadley, '26). The superficial layer of the epidermis undergoes cornification. In this type of graft, invasion of blood vessels may occur from any point on the surface.

In a second type of graft, vascularization is effected at one more or less localized point of the graft, the remainder of the donor tissue being completely surrounded by a tunic of stratified connective tissue and a more or less complete epidermal layer (figs. 4 and 5). Limb-bud grafts are frequently of this type. Primarily attachment evidently involved only the exposed mesenchyme of the transplant which adhered to the mesothelium of the omentum. (Vascular elements first entered the transplant through this primary fusion zone.) A narrow space, probably containing fluid, often separates the epidermis from the enveloping host tissue. Other cases show the host tissue in intimate contact with the graft. In such grafts epidermal epithelium is in the form of flattened sacs whose cavities are filled with blood cells (fig. 5).

B. Changes within the tissues of the graft

In addition to the morphological and histological differentiation of the implanted primordium, several conditions are consistently present. There is an extravasation of host blood in all stages and types of grafts, which is especially prominent in the immediate vicinity of the differentiating tissues, and which takes the form of free blood cells scattered in large

quantities throughout the graft tissue, in all lumina, and in the immediately adjacent connective tissue (host). This phenomenon is most evident in grafts of the otocyst and optic primordia, least so in those of the limb buds. The origin and fate of this material and its significance will be taken up below. There is a marked eosinophilia throughout the tissues of the graft and in the immediately adjacent connective and fat tissues. It is especially abundant in the peripheral regions of the graft. Large and small lymphocytes are common, while in portions of the peripheral regions round-cell infiltrations have occurred to form small isolated nodules. The latter usually are associated with a small blood vessel. In both types of graft, macrophages which had been previously stained by intra-abdominal injections of lithium carmine in Ringer-Locke solution were found in or along the periphery, never within the graft. It would be reasonable to suppose that if any conspicuous invasion had occurred these macrophages would have been involved. After transplantation, further transformation of macrophages normally occurs, and as these are not marked with dye, it is difficult to identify them in fixed preparations unless they have been actively ingesting material. Such cells are not conspicuous in the younger grafts. They do become quite numerous in the older growths where evidences of degeneration are prominent. There occurs a marked fibroblastic reaction on the part of both the mesenchyme of the transplant and that of the omentum. The mesenchyme of the transplant gives rise to broad bands of fibroblasts which appear to isolate parts of the transplant, while that of the omentum forms similar bands that encapsulate the graft as a whole and in addition often unite adjacent grafts into one mass. In this way, host tissue, in addition to blood vessels, is found in the interior of the compound grafts. While there is some evidence of fibroblastic invasion in those regions where donor and host tissue have fused, it is not necessary to postulate any marked invasion of host mesenchyme. It appears that the only conspicuous invasion of the graft is by the proliferation of those

omental blood vessels which are in its immediate vicinity. Considerable fat accumulates around the periphery of the graft as it grows older, especially if it is located in the outer regions of the omentum where fat normally is found. This depends greatly on the diet of the animal.

Thus there seem to be several facts which show that no marked invasion of host mesenchyme occurs. These are: 1) the failure to find previously stained macrophages within the graft; 2) the absence of any fatty tissue except in the peripheral regions; and, 3) the inability to trace fibroblastic invasion from the peripheral regions into the interior of the graft. In general, any host cells which reach the interior of the graft are carried there by way of the invading blood vessels.

C. Extravasated blood

Of especial interest is the amount of extravascular blood which is present throughout the tissue spaces of the graft and often fills all lumina (fig. 3). The present observations depend entirely upon fixed material, but it seems likely that the presence of the blood cells is the result of the distention of newly proliferated capillaries as they make their way through the donor tissue. Arterial pressure, as a result, forces the blood cells, chiefly erythrocytes, through the delicate endothelial walls of the capillaries to produce extensive hemorrhages. In this way cells normally flowing in blood channels become intermingled with graft tissue. This extravasated blood is present from the very earliest stages to the oldest grafts that were recovered—a time interval of approximately twelve days. Grafts recovered two to three days after transplantation, the transplants being in the earlier stages of incorporation, are generally gorged with these extravascular erythrocytes, among which are large and small lymphocytes, together with a large number of eosinophils of varying stages of differentiation. As the grafts grow older, the number of blood cells increases enormously, until in some cases, where large epithelial cysts are present, the cavities may be packed with them.

The blood cells are abundant wherever space permits their presence, as in connective tissue and in brain tissue, which frequently presents a very disorganized condition in the grafts. They occur in the precursor of the perilymphatic space of the membranous labyrinth, into which they have penetrated by breaks in the enclosing cartilaginous walls, and in the lumina of ear and eye grafts. However, they are never found in more compact tissue, such as the epithelium of eye and ear grafts, and in cartilage.

In the interior of the graft and in the organ cavities the erythrocytes appear to be in a healthy condition; no coagulation of the blood is noticeable; nor is there any evidence to show that the cells have undergone disintegration. Furthermore, macrophages are conspicuously absent, except in the periphery of the grafts, where some phagocytosis of erythrocytes and granular leucocytes is evident. These observations indicate either a pronounced ability of mammalian erythrocytes to survive for long periods in embryonic tissue spaces or the possibility of the removal of the erythrocytes by some other means than phagocytosis. If the latter is the case, there may be a sluggish movement of blood through the tissue spaces of the graft. This has not been demonstrated in living grafts at the present time.

Apparently very little attention has been paid to this phenomenon in chorio-allantoic grafts. In several papers (Danchakoff, '18, and Hoadley, '24) mention is made of the presence of extravascular blood cells, often occurring in clumps which seem to lack confining walls, while in others there is no mention of this condition. It would seem that this phenomenon is of such common occurrence that it has been taken as a matter of course. Yet it appears very strange that erythrocytes are able to exist in an apparently healthy condition in tissue spaces and cavities of organs without undergoing coagulation, degeneration, or phagocytosis. It is commonly known that rabbit blood desired for culture purposes must be kept free of tissue juices to avoid coagulation. Furthermore, the addition of embryo extract to heparin-

ized blood will coagulate it, the time required for coagulation depending upon the amount of extract which is added. Danchakoff states that the outer epithelium of the chorio-allantoic membrane is destroyed in part by hemorrhages and the erythrocytes are removed in large numbers by other cells exhibiting phagocytic activities. Hoadley ('24) has noted extravascular erythrocytes in grafts of nervous tissue, especially brain. He says that there is present an infiltration of blood cells "which do not appear to be located in any organized vessels" (p. 295). "In places there are what seem to be large sinuses filled with blood cells" (p. 297). "Even in the mesocoele there are large groups of blood cells though no organized vascular walls appear around them" (p. 308). This clearly shows that the presence of extravascular blood cells is also characteristic of chorio-allantoic grafts.

Clark and Clark ('26), investigating the fate of extruded erythrocytes in living *Hyla* larvae, find that these cells are removed by lymphatic capillaries and tissue phagocytes. The latter are pigmented mononuclear wandering cells, similar in their reaction to the histiocytes of the mammal. By exerting moderate pressure on the tail, small hemorrhages were produced at the tips of blood capillaries or in the newly formed loops of young blood vessels without any visible effect on the other cells of the region. This produced a hemorrhagic condition similar in a small way to that noted in the omental grafts. The newly formed endothelium proved to be more delicate and more easily injured than that which had been present for some weeks. They find also that a period of fifteen to twenty hours must elapse before a red cell can be phagocytosed, suggesting a loss of immunity to phagocytosis on the part of the cell after this time. The lymphatic capillaries are also attracted by the extravascular erythrocytes, providing the blood cells are not too far removed, and send out fine processes by means of which the erythrocytes are slowly returned to the main trunk. In very young tadpoles this capacity for sending out sprouts, by means of which the erythrocytes are returned to the circulation, is also a property of capillaries not more than five days old.

Sandison ('28), in his studies of tissue growth within the transparent chamber inserted into the rabbit's ear, has observed a similar weakness on the part of advancing capillaries. Hemorrhages mark their growth and appear to precede them as a solid red line of closely packed erythrocytes. Moreover, the endothelial sprouts which develop from the loops of newly formed capillaries are often distended by a sudden increase in blood flow. Through the distended and weakened endothelial walls, the erythrocytes are squeezed into the tissue spaces. Such an extravasation accounts for the density of the red line usually seen in the distal part of a newly growing region. Sandison considers that this regularly accompanies the growth process. As these vessels become a few days older the endothelium is no longer distended and no further extrusion of blood cells is observed.

As the omental grafts become older the amount of extravasated blood may decrease perceptibly, but this is not always the case. Such a condition would apparently indicate a decrease in activity on the part of the invading capillaries such that a strengthening of the capillary walls would prevent the escape of the cells. Sandison ('28) finds that a few days is a sufficient interval for this to occur. Yet other grafts are even more gorged with blood elements at these older stages. In some few cases most of the graft tissue has disappeared, leaving only a huge mass of loose blood elements restrained by connective tissue of the graft and the encapsulating fibroblastic layers. Many features about graft development remain to be explained, as too little work has been done on this phase of the subject thus far. Chief attention has been paid to the end result, very little to graft development. The present observations contribute little to the solution of the manner in which extravascular cells are returned to the blood stream, if indeed they are. Removal by active macrophages cannot be demonstrated, at least in the interior of the grafts; neither is there any perceptible evidence of the disintegration of these cells. The most that can be said is that the extravasated blood cells which accumulate during

graft development are apparently able to survive for a considerable length of time in the tissue spaces and organ cavities of grafts. They may be slowly removed by the activity of young haemal and lymphatic capillaries.

D. Retrogression of the graft

The size of the transplanted piece appears to determine in part the success of the graft and consequently the differentiation of its constituent parts. In the course of the present study thirds of ten-day rabbit embryos were employed in the initial survey. Differentiation in such large pieces was very limited, but when small isolated primordia were used instead of thirds of embryos, the amount and degree of differentiation attained by these transplants were far more extensive. The larger the transplant, the longer the delay in complete vascularization, with the result that those areas farthest from the invading blood vessels fail to secure the necessary food supply and elimination of wastes. Such areas soon become necrotic and this must exert a profound influence upon other parts of the transplant, resulting in early degeneration. Surface tissues such as epidermis differentiate well, because vascularization is immediate; while cartilage persists, due to its very nature. Thus the size of the transplant very probably accounts for much of the poor differentiation secured in the experiments where large pieces of tissue were used. Normally where vascularization has proceeded rapidly from the beginning and subsequently keeps abreast of graft growth and differentiation, all parts of an active graft are in a healthy condition, exhibiting no necrosis or phagocytic activity. This is possible only if the invading capillaries have been able to reach quickly all parts of the transplant.

The toleration of the omental tissues exhibited toward transplants of the same species is indicated by another line of evidence. It is indirect, but nevertheless conclusive, showing that if the transplant is incorporated and vascularized, the phagocytic activities of the connective tissue are at first definitely inhibited. This evidence comes from the effort to

graft isolated primordia of mouse and chick embryos upon the omentum of the rabbit. In every case the transplants failed utterly to become incorporated, no trace of a graft being found following such an operation. The defense reactions of the omentum apparently were mobilized very early, resulting in the total elimination of mouse and chick tissue. The literature on heterospecificity has been admirably summarized and discussed by Loeb ('30).

As long as the subordinate parts of a successful graft are actively proliferating (a condition indicated by the number of mitoses present) and differentiating, the omental tissues tolerate the presence of the embryonic tissue, furnishing it with the materials necessary for continued life. No effort has been made to determine the length of life of a well-incorporated graft. Grafts showing various stages in degeneration, such as extensive necrotic areas containing small multinucleated giant cells and macrophages, are found at the varying times when the grafts are recovered, while others secured from the same omentum are still in a healthy condition. The former are very probably the result of numerous unfavorable conditions encountered during development, such as retarded or scanty vascularization, excessive accumulation of fat, injury at the time of transplantation, or restricting pressures and tensions from the surrounding tissues. Necrotic areas are not found in growing, well-incorporated grafts.

As has been mentioned previously, successful eye and ear grafts attain their maximum size and differentiation between the tenth and twelfth days (limb-bud grafts much later), at which time cell proliferation has practically ceased. This cessation of graft activity marks the initial onset of degeneration. The walling off of the graft by host activity continues and tends to reduce the blood supply by pressure exerted upon the capillaries. The phagocytic tendencies of the omental tissues are now marshalled for the removal of the transplanted tissue.

One of the first tissues to disappear is the epithelium of the eye and ear grafts. The nuclei of the epithelium become pycnotic, while in some cases there occurs a breakdown of cellular walls to form syncytial masses of considerable area containing lightly staining vesicular nuclei. The epithelium is further disorganized by the invasion of wandering cells that appear to break up the formerly compact epithelium into smaller areas. This is subsequently followed by marked necrosis. Small multinucleated giant cells together with fibroblasts and granular leucocytes are conspicuous in such degenerating regions.

Long after the epithelium of the sense organs has been completely removed, cartilage, muscle, and epidermis appear to be in a healthy condition. Of these, cartilage is the last to be removed. Ear grafts are excellent examples of this. The epithelium of the membranous labyrinth may have entirely disappeared, while the enclosing cartilaginous wall shows no evidence of degeneration. In such cases the cavity of the capsule is filled with a network of connective tissue and fibroblasts, so that it furnishes an excellent situation for the study of the activity of these cells. Limb-bud grafts also have a relatively long existence; in fact, it is only in the later stages that perichondrial and endochondrial bone is developed.

The results of these experiments tend to show that the loose areolar connective tissue of the omental wall reacts in a definite manner to the presence of the transplant. The transplant is incorporated and is vascularized by invading proliferations of host capillaries, in this way assuring the necessary food supply and elimination of waste material essential to growth and differentiation. Accordingly, the omental tissues appear to tolerate the transplant, all phagocytic tendencies being practically inhibited as long as the graft is actively growing. Once growth and differentiation have ceased, degenerative changes ensue. The omental wall furnishes a suitable medium for the study of the capacity for independent self-differentiation possessed by isolated primordia of the rabbit embryo.

SUMMARY

1. Isolated primordia introduced into the omental bursae of adult male and pregnant and non-pregnant females are incorporated by the loose areolar connective tissue of the omental wall. The analysis of the process of incorporation is based on a series of grafts recovered from the third to the fifteenth day of growth.

2. The smaller the transplant, the better the resultant differentiation of its constituent parts. Large pieces such as thirds of embryos fail to secure the necessary blood supply, with the result that necrotic areas, often of considerable extent, appear within the graft.

3. The grafts when recovered are usually large, somewhat spherical bodies, often of a deep red color from extravasated blood cells which fill the tissue spaces and organ cavities. Others may be so small, colorless, and surrounded by fat as to be detectable only by gently pressing the tissue between the fingers.

4. Neither sex nor age of the host exerts any perceptible influence either upon the process of incorporation or upon the degree of differentiation exhibited by the graft. The use of an anesthetic (ether) likewise does not retard graft development.

5. The transplant becomes completely surrounded by host tissue by the third to fifth day. The reaction between host and donor tissues is not a limited phenomenon, but continues throughout the life of the graft, finally culminating in its removal. Where two or more primordia are adjacent, they are frequently incorporated into one large compound graft.

6. A preliminary adhesion between transplant and omental mesothelium is followed by a thickening of the loose areolar connective tissue in the region of contact, the breakdown of the mesothelial layer, the overgrowth of the transplant by host tissue, and the invasion of the transplant by host capillaries.

7. Grafts generally attain their maximum size and differentiation by approximately the tenth to the twelfth day of

growth. The omental tissues appear to tolerate transplants of the same species, all phagocytic tendencies being replaced by nutritive and protective ones, as long as the graft is actively growing and differentiating. Once these processes have ceased, degenerative changes ensue involving marked necrosis followed by phagocytosis. Transplants of mouse and chick primordia failed utterly to 'take' in the omentum of the rabbit.

8. The extravasated blood in the graft probably has its origin from the proliferating capillaries. Arterial pressure forces the erythrocytes through the delicate endothelial walls of the distended capillaries and they become mingled with the tissues of the grafts. The fate of these extruded cells is problematical.

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PLATE 1

EXPLANATION OF FIGURES

All figures are photomicrographs.

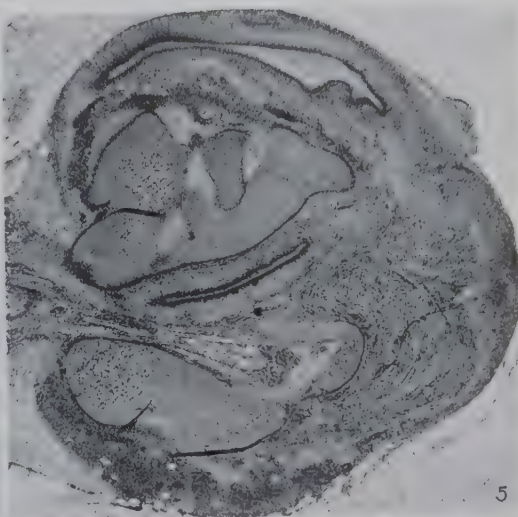
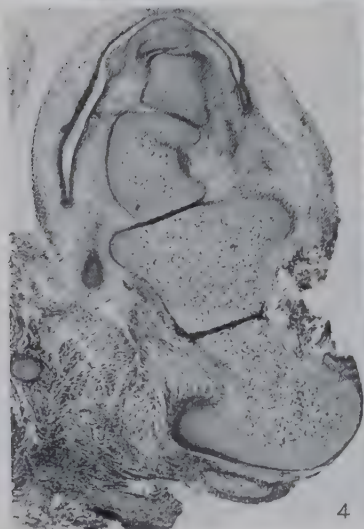
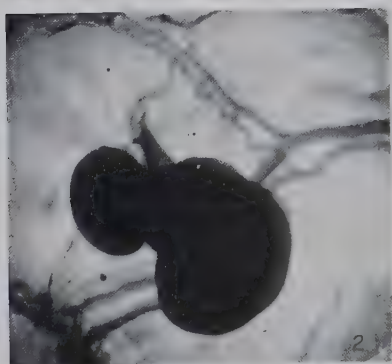
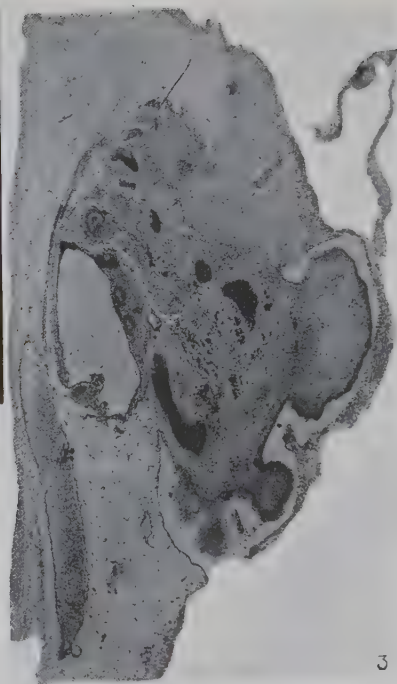
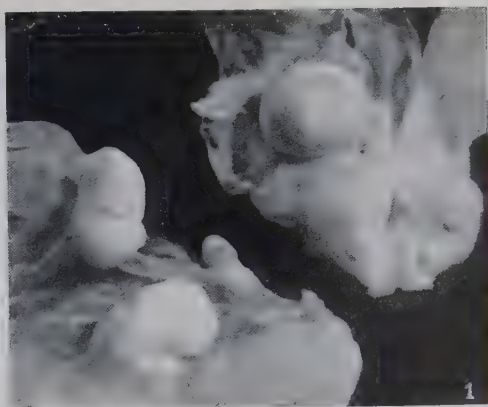
1 Omental grafts of ten days' growth, photographed in alcohol by reflected light. $\times 5.5$.

2 Omental graft photographed in cedar oil by transmitted light to show vascularization. $\times 8$.

3 Cross-section of young compound graft of six days of growth, showing the early stages of incorporation. $\times 30$. The omental tissues have thickened and have grown around the differentiating primordia. The break in the enclosing wall is an artifact due to sectioning. Extravasated blood is present in the tissue spaces and lumina. Note the large host blood vessel in the lower part of the figure.

4 Longitudinal section of a graft of an eleven-day anterior limb bud recovered eleven days after transplantation. $\times 30$. The graft is attached by a broad stalk to a large compound graft. Note the well-differentiated epidermis partially enclosing the distal end of the limb cartilages. Part of the incorporating tissue on one side has been torn away during sectioning.

5 Cross-section of a graft of a ten-day anterior limb primordium recovered after thirteen days. $\times 30$. The graft surrounded by fat is attached to the omental wall by a narrow stalk, by way of which vascularization has occurred. The epithelial cysts and tissue spaces of the graft contain extravascular blood cells.



THE FORMATION OF FAT IN THE HEPATIC CELL

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FOUR FIGURES

One of the authors has recently described the formation of fat in the hepatic cell of the white rat, basing the account upon sections prepared by Regaud's formol-bichromate, iron-alum-haematoxylin method for mitochondria (Smith, '31). All of the animals studied at that time were killed twenty-four hours after feeding, and under that condition, the mitochondria in many of the individuals of more than eight days of age appeared to be homogeneous bodies, being stained throughout by the haematoxylin. This was true of all types of chondriosomes. However, in all individuals from seventeen days of gestation until eight days after birth, and in part of the adults, some of the spherical mitochondria developed what appeared to be minute spherical cavities in their center. The character of these cavities in the hepatic cells of all fetuses and newborn of less than two days of age did not become apparent, because they remained relatively small and were not in any case freed from their mitochondrial covering. After the latter age, however, these vacuoles enlarged greatly, and, concomitantly, their mitochondrial covering became thin and fragmented by a process of gradual furrowing from the outer surface, resulting in the formation of short crescentic rod-shaped mitochondria and the liberation of the vacuolar-appearing structure in the ground cytoplasm. After this occurrence, it was evident that these structures were clearly the spherical vacuoles left by the dissolution of fat globules, a characteristic feature of hepatic tissue prepared by Regaud's method.

This observation was interpreted as follows:

The fact that fat globules are formed in the substance of spherical chondriosomes to be later liberated in the ground cytoplasm by the fragmentation of their mitochondrial covering must be interpreted in one of two ways, either the mitochondria serve as a storage place for fats or act as catalysts stimulating a synthesis of fats from the fatty constituents. The non-diffusibility of fats makes the former seem very unlikely. Thus, I am led to conclude that the above description constitutes very strong evidence for the catalytic nature of mitochondrial activity, at least in reference to fat formation.

Prolonged and intensive study of liver sections makes it possible for one to accurately identify structures of this kind, as well as glycogen vacuoles, without employing any micro-chemical tests, so that there is little doubt as to the actual character of the above-described vacuoles. At the same time one must recognize that the far-reaching significance of these observations makes it desirable to establish unequivocally the chemical nature of the material within such chondriosomes. As this, at least theoretically, can be done by the use of mitochondrial techniques that require fixation in osmium tetroxide, followed by a mitochondrial stain that is some color other than black, we have deemed it desirable to study this problem by using a number of different methods of fixation and staining. We have again used the livers of rats as our principal material. Some as yet incomplete studies by the junior author show that the initial steps in fat formation are found in the hepatic cell of rats eight hours after feeding a pure diet of cane sugar. For a few ensuing hours the fat globules increase in number and size. We have accordingly taken our material eight hours after the animals had eaten, as it was desired to get the early stages in the elaboration of this reserve product. A block of tissue from each liver was fixed in each of the following solutions, so that accurate comparison of results would be possible. Some incidental observations have also been made on the hepatic cells of the cat. The data obtained by the use of several of the techniques employed are so clear-cut that it seems desirable to make a separate report on fat formation in the liver at this time.

OBSERVATIONS

Hepatic tissue of the rat

Schridde's fixation and Altmann's stain. As a general fixative for liver tissue Schridde's can hardly be regarded as satisfactory. The cells seem inclined to swell to the limit of available space, so that ordinarily no vestige of capillaries can be seen in material taken eight hours after feeding. Neither is the ground cytoplasm as well preserved as by a method such as Regaud's; that is, it appears to have contained very little coagulable substance. It is also true that the efficacy of this fixative in the preservation of normal mitochondrial form is poor. Its penetration is good and both filaments and spheres are found throughout the section, even when the tissue is fixed in relatively large pieces. However, the contour of the chondriosomes is not smooth, as in most fixations. Both spheres and filaments, as well as intermediate shapes, have their periphery roughened by the formation of papillary eminences that give all of them somewhat of a burr-like appearance.

Despite all of these bad qualities, this fixative yields the most decisive of evidence on the present question. The fat present in the hepatic cell of a rat killed eight hours after feeding is all in the form of relatively small globules, which are increasing rapidly in size, judging from material taken a few hours later. These small globules are, of course, stained black by the osmic acid, and, substantiating the previous conclusions of Smith ('31), are very clearly surrounded on all sides by mitochondrial substance (fig. 1). Practically all of the small fat globules observed in this material are located in the center of mitochondria, much more frequently in spheres than in filaments, though an occasional one is seen free in the ground cytoplasm.

In case of some of the larger globules the mitochondrial covering is unevenly stained by the fuchsin, seemingly being broken up into areas of deeply staining material, with the intervening spaces a light pink. This gives an appearance somewhat similar to the furrowing that precedes fragmenta-

tion in Regaud-fixed sections. However, the actual separation of the mitochondrial covering from the fat cannot be observed.

The sharp though light yellow of the ground cytoplasm, the black of the fat, and the dull red of the mitochondria leave no room whatever to doubt the accuracy of the above statement. Of course, there is another aspect, namely, whether the chondriosomal material actually entirely encloses the fat or merely forms a band around it. Our selection of the former as true is based upon two considerations: first, there is no

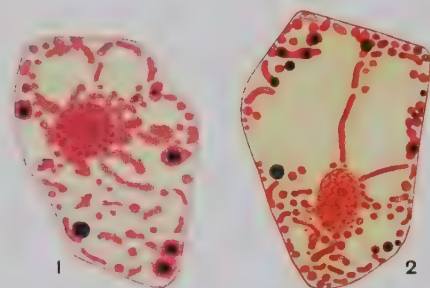


Fig. 1 Hepatic cell from the middle portion of a lobule. Note the fat globules enclosed in mitochondria, and the irregular contour of the latter. Schridde fixation and Altmann stain. $\times 1600$.

Fig. 2 Same type of cell as that shown in figure 1. Observe that the mitochondria are even in outline and that most of the fat globules are free in the ground cytoplasm. Altmann fixation and stain. $\times 1600$.

conceivable force that would govern the orientation of the bands so that they would all appear circular in the sections; secondly, very careful focusing above and below the plane of the fat globule always discloses a red body. The cell wall is the only exception to the perfection of differentiation, the borders between cells always showing the fuchsin.

This fixation can be followed very successfully by Altmann's acid fuchsin-picric acid stain. The recommended method of using hot iron-alum-haematoxylin would not serve the present purpose because it would not give the desired differentiation of cytoplasm, mitochondria, and fat.

Altmann's method. Altmann's method of fixation is similar to Schridde's in its general preservation of liver tissue, that is, in the obliteration of capillaries, in the sparseness of the coagulable material of the ground cytoplasm, as well as in penetration. At the same time the reaction of the mitochondria is quite different, the contour of all types being exceptionally smooth. In fact, in this respect the two fixatives would be opposite extremes, even if quite a number of methods are being considered. The size of the mitochondria is about equal in Altmann and Schridde sections, though they may be a trifle larger in the latter.

The high percentage of osmic acid in this fixative makes the usual immersion for twenty-four hours adequate for the clear-cut staining of all fat, and again we find support for the general conclusion previously drawn. However, with respect to details, the topographical relationship of fat to the mitochondria is quite different from what it is in Schridde material. There are a great many more globules that are free in the ground cytoplasm, about three-fourths of those observed, and most of the larger ones that have any close spatial relationship to mitochondria are not completely surrounded by the latter, but are merely partly enclosed in broad band-like chondriosomes that do not entirely surround them in any plane. Another difference is in connection with the filamentous mitochondria. These are seldom observed to contain fat in Schridde sections, but in Altmann's most of the fat globules that are entirely enclosed in mitochondrial substance are found in filaments, but only relatively small ones can be so observed. In some cases, two entirely separate globules occur in a single filament. Unlike Schridde sections, the mitochondrial covering of fat globules is always homogeneously stained (fig. 2).

So far as the differential nature of the stain is concerned, the results of this technique are even more easily interpreted than in the case of Schridde's, as the ground cytoplasm is more sharply delimited from the mitochondria. We modified the usual procedure by destaining in cold picric acid, remov-

ing slides from the latter before they were adequately destained, and studying them after two or three days' exposure to indirect sunlight in the laboratory. Immediately after staining, the differentiation is not so sharp, and after prolonged exposure to light the necessary depth of color is lost. The coloration of the mitochondria is more of an orange-red than in Schridde sections.

Benda and Champy-Kull fixation. The general character of the Benda and Champy-Kull methods is well known and need not be reviewed in detail here. However, mention should be made of their pronounced distortion of mitochondrial form by way of converting all types into spheres, in all cells except those in a few peripheral layers, even in small blocks of tissue, as our interpretation of the results of their use is based partly upon that feature.

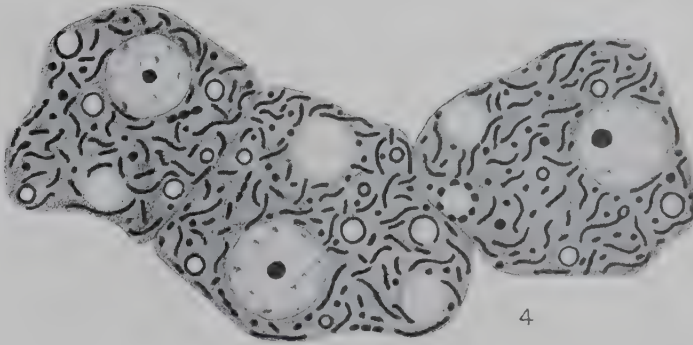
When these two fixations are followed by Benda's stain of alizarin and crystal violet, the fat can be very clearly differentiated from the mitochondria in the interior of the block, where the cells are enlarged and the chondriosomes more widely spaced, but not a single fat globule was observed to be enclosed in a chondriosome, and in most cases they were not even in contact with them. The same is true when the acid-fuchsin stain is used.

Considerable difference is found in the well-fixed peripheral portion. Here the cells are much smaller and the mitochondria are consequently closely crowded. As a result of the crowding and the darkness of Benda's stain, it is difficult to clearly distinguish fat and mitochondria, when that stain is employed. Naturally, it is also impossible to discern with certainty any fat globules completely enclosed in chondriosomes. However, when the sections are stained with fuchsin, a few fat globules, perhaps 5 or 10 per cent of those observed, can be seen to be surrounded on all sides by mitochondrial substance. Thus we can hardly consider that the data obtained from either of these techniques clearly substantiate the results of the use of those described above.

Regaud's method. The appearance of the mitochondria containing fat vacuoles in Regaud sections has been adequately described previously (Smith, '31). There is, however, one slight correction to be made to that account, namely, that after fragmentation of the mitochondrial covering of the fat vacuole has started there is sometimes a retraction of the chondriosomal material so that the sphere becomes incomplete, though this condition is seldom found. Figure 3 is taken from the same liver illustrated in figures 1 and 2.



3



4

Fig. 3 Same type of cell as shown in figures 1 and 2. The mitochondria are more closely crowded than in the above figures and a few hollow spherical ones can be observed. Regaud fixation and iron-alum-haematoxylin stain. $\times 2560$.

Fig. 4 Three cells from the midregion of a hepatic lobule of the cat. There are numerous hollow spherical mitochondria, some of which are fragmenting, and some fat vacuoles free in the ground cytoplasm. Regaud fixation and iron-alum-haematoxylin stain. $\times 2560$.

Hepatic tissue of the cat

One of the authors has previously described the mitochondria of the hepatic cell of the cat (Kater, '31). It is noteworthy that in the material used for that study, which was prepared by Regaud's method, no hollow spheres, such as those described by Smith ('31) in the rat, were found. In that work the cats, sixteen in number, were killed twenty-four hours after feeding and were in excellent condition, appearing very healthy and normal in weight. Since that time another group of cats, seventeen, has been used in investigating another problem concerning hepatic mitochondria, and, incidentally, has afforded some very good data on fat formation.

The latter group, unlike the first, consisted of cats that were in very poor condition. They had been in the animal house for several months, and has been rather scantily and irregularly fed, to the extent that they were somewhat emaciated. Their livers had become more fatty than usual, and, when killed twenty-four hours after feeding, early stages in the formation of globules in the interior of mitochondria were abundant (fig. 4), more so than Smith ('31) found them in rats.

Unfortunately, all livers taken from these cats were fixed in Regaud's, so that nothing in the nature of a microchemical test for fat has been employed. However, the spherical vacuoles observed within the mitochondria are so similar to those found in the rat, where the fat has been clearly identified, that there is no doubt as to their nature.

In the very early stages of fat formation one can observe an extremely minute vacuole in the interior of either spheres or filaments. Their occurrence in the latter distinguishes the cat from the rat, in which they appear only in spheres, after Regaud's fixation. These small vacuoles are always completely surrounded by mitochondrial substance, in the horizontal plane, and careful focusing shows that the spheres are also complete above and below the fat vacuole, just as in the case of the rat.

In the later history of these structures, a pronounced difference is seen in the two animals studied. In the cat the complete mitochondrial covering of the fat globule breaks and apparently contracts, so that the latter is only partly enclosed in a broad band-like chondriosome that completely encircles it in one plane. This condition can be detected because of the oval cavity that is observed at some levels in some of the mitochondria and by actual observation at upper and lower focal planes.

The separation of the mitochondria from the fat differs in the two animals also, since the curved rods characteristic of the rat are entirely absent in the cat. In the latter only small spheres are found. These form a circle around the fat and gradually migrate away from it.

Nine of the seventeen cats received bile either orally or subcutaneously and three were killed in a hyperglycemic state. Neither of these conditions perceptibly affected the number or character of vacuolated mitochondria.

DISCUSSION

Variability of structure after different techniques

Considering all of the above methods for the demonstration of mitochondria, it is evident that they fall into two groups, with Schridde's, Altmann's, and Regaud's forming one and Benda's and Champy-Kull's the other. The first group of fixatives so preserves hepatic tissue that the small fat globules are completely enclosed in chondriosomes; the second so seldom preserves this condition that we can reasonably regard it as not doing so at all. Unfortunately, every cytological problem is accompanied by the question of artifact, and we have it here. Which of the two groups preserves the actual living state and which is producing artifacts? The very poor quality of the Benda and Champy-Kull methods for general fixation and their extremely low ability to penetrate a compact tissue, such as the liver, for more than a few layers of cells would incline us to be favorable to the normalcy of the picture produced by the other methods. Perhaps the most convincing

evidence for this same conclusion comes from the use of the Altmann method, where perfectly normal short filaments with smooth contour are seen to contain fat globules. We can hardly conceive of a dispersal of mitochondrial substance, followed by its precipitation around fat globules, resulting in the formation of mitochondria of this character, and this is the only way in which the observed condition could be interpreted as artifact. The picture presented by Schridde material could more conceivably be of this nature, because the mitochondria are seldom seen even in outline and might reasonably be regarded as a haphazard precipitation, if it were not for the fact that practically all small fat globules are so enclosed. In addition, if it were a matter of precipitation, there is no reason why the large fat globules should not likewise be found within mitochondria. Thus, it would seem that we are justified in considering that the Schridde, Altmann, and Regaud methods give us a true picture of the early stages in the formation of a fat globule, and that the Benda and Champy-Kull methods fail to give this picture because of their distortion of this feature of cellular structure, at least in hepatic tissue.

The fact that fat is found in both filaments and spheres in Altmann and Schridde sections, and only in spheres in Regaud sections of the rat liver, introduces another question of artifact. The mitochondria are much larger after Altmann's technique than they are after Regaud's, so that we are inclined to believe that the latter fixative causes a shrinkage of mitochondria which might conceivably cause the ends of filaments to contract against the distended portion, where the fat is located, thus giving the appearance of a sphere to the structure. There is apparently no shrinkage of mitochondria in Schridde's fixation, but the papillary nature of the surface of these elements would suggest at least some slight distortion, and might explain the scarcity of fat globules within filaments.

Rôle of mitochondria in fat formation

The quotation from Smith ('31), given in the introduction, expresses, we believe, the most reasonable interpretation of the above-described relationship between mitochondria and fat globules. It might be well, however, to enlarge somewhat upon that statement of it. It seems possible that the diffusible products of hydrolysis of fats may either diffuse into the chondriosome and, under influence of the latter, become reconverted into fat, or first become a part of the mitochondrial substance, later to be converted into true fat. At present, we have no evidence reflecting upon these two possibilities, and consequently, cannot select either as true.

A solution of that question is not essential, however, for a general interpretation of the functional significance of mitochondria. The important facts are that fat globules are formed within a chondriosome, and the latter retains its integrity as a chondriosome—in fact, becomes multiplied at the end of the process. Thus, we see nothing to deter us from concluding that the function of the mitochondrion is of the nature of a catalyst. Any attempt to attack this interpretation upon the claims that the fatty constituents may have first become a part of the mitochondrion is merely of polemical interest, because, whether the constituents of the fat become a part of the chondriosome or merely diffuse through it, a synthesis in the interior of a mitochondrion is still involved.

Although this evidence constitutes a very clear-cut solution of the problem of the early development of the fat globule in the hepatic cell, nevertheless it is not of such a nature as to constitute a refutation of the idea that mitochondria become bodily transformed into fat, even though it would incline one to be skeptical of such an interpretation. Accordingly, a discussion of the papers dealing with that phase of the question is not warranted. We realize also that this solution refers only to the early development of a fat globule. After the mitochondrial covering is lost, the globule continues to increase in size, and when it is utilized it must be hydrolyzed

without the presence of a complete mitochondrial sheath, so far as is now known. The present observations elucidate in no way those phases of fat metabolism. Likewise, as already stated by Smith ('31), the observations do not reflect upon the many other problems concerning mitochondrial form and function.

Noël ('23) concluded that fat is formed in the hepatic cell of the mouse under influence of the mitochondria. This was based upon the observation that hypertrophied spheres were surrounded by a ring of small spherical fat globules, similar to the reverse relationship observed in the cat after the hollow sphere has fragmented. He also observed a ring of black material around the mitochondria after Benda's fixation and stain. Our preparations by this method suggest that the osmic acid is occasionally rather irregularly reduced so that a blackening of the periphery of all bodies in the cell is effected.

This same worker also observed spherical cavities in spheres (grains à coque) and in the ends of filaments in Regaud sections. He suggested that these probably were formed in connection with the secretion of bile, as they appeared to increase after feeding this substance. It has been noted above that the cats used in the present study do not substantiate this observation with reference to the effect of chologogues. Our experience with the various techniques used in this work indicates that one should not be surprised at Noël's failure to determine the true nature of these elements, as both Benda's and Champy-Kull's methods fail to demonstrate the intramitochondrial fat, and Altmann's, though furnishing some very clear-cut examples, requires considerable study before it is convincing. Similar hollow spheres have also been observed by Clark and Hair (unpublished) in the hepatic cell of the hibernating frog. However, the fragmentation of the mitochondrial covering was not observed.

Some other papers dealing with mitochondria in various tissues indicate that the authors have observed structures

similar to some phase of the mitochondria associated with fat globules described in the present account. Many of these are difficult to interpret and cannot well be discussed. However, D'Agata ('13) has undoubtedly seen the hollow spheres found in Regaud material in sections of the pancreas taken from animals in diphtheritic intoxication. He has accurately interpreted them as bodies active in fat formation, although his demonstration of this fact was not entirely satisfactory, because of his failure to successfully combine specific fat and mitochondrial techniques. Da Costa ('13) showed, by the use of Benda fixation, that fat globules are sometimes enclosed in mitochondria, but interpreted this as a stage in the conversion of mitochondria into fat globules, the transformation being initiated in the interior of the mitochondrion. Consequently, the true significance of the observation has not been recognized.

It is quite possible that in studying this same problem in other tissues the Schridde-Altmann method would not yield the same clear-cut results that it does in the case of the liver. Accordingly, one should endeavor to use as many similar methods as possible.

SUMMARY

After fixation of liver tissue of rats eight hours after feeding cane sugar, according to the methods of Schridde and Altmann and staining according to Altmann, the early developing fat globules are seen to be embedded within the substance of mitochondria. This affords a very concrete verification of the interpretation of Regaud sections previously made by one of the authors.

The Benda and Champy-Kull methods seldom show this feature of cellular structure, probably because of distortion of the normal state.

Although one group of sixteen cats has been previously studied without finding any similar stages in fat formation, another group of seventeen has recently been used in which all livers had an abundance of such structures.

It is pointed out that these observations constitute very strong evidence for the catalytic nature of mitochondrial activity, with reference to fat formation.

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HETEROPLASTIC TRANSPLANTS OF DUCK KIDNEY TISSUE ON THE CHORIO-ALLANTOIC MEMBRANE OF THE CHICK IN CONJUNCTION WITH ADULT CHICKEN SPLEEN GRAFTS

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INTRODUCTION

This paper reports a series of experiments in which an attempt was made to induce a precocious species specificity in the developing chick embryo by means of grafts of adult chicken spleen. Murphy ('14, '26) transplanted hashed embryonic and sarcomatous tissue of the rat to the chorio-allantoic membrane of the chick and found that it grew well until the host embryo had reached an age of eighteen days of incubation. The grafted tissue then became necrotic and infiltrated with lymphocytes. However, if either adult chicken spleen or bone marrow was grafted in conjunction with the rat tissue, necrosis and lymphocytic infiltrations occurred at a very much earlier age than the eighteenth day of incubation. Thus he concluded ('14, p. 521) that—

. . . . the chick embryo offers suitable conditions for the growth of implanted tissues, whether these be embryonic or adult, of the same species or a foreign one. The chick at about the time of hatching develops a defensive mechanism against the tissue of a foreign species. This resistance can be supplied to the embryo in the early stages if grafts of adult spleen or bone marrow are implanted. Under these conditions the embryo exhibits the same resistance to foreign tissue as does the adult, and presents the same histological manifestations about the graft.

Sandstrom ('32), in an attempt to correlate the time of the appearance of species specificity and differentiation, grafted small pieces of metanephric tissue from ducks vary-

ing in age from thirteen-day-old embryos to adults to the chorio-allantoic membrane of the chick. He found that undifferentiated nephrogenous tissue grew and differentiated into apparently normal kidney structures, but already differentiated metanephric tissue would not grow. Instead, it became necrotic. This he found true even though the host was less than eighteen days of incubation at the time the grafts were removed. His results, however, gave no conclusive evidence that the failure of the kidney tissue to grow after it was differentiated was due to the development of a species specificity in either the host or donor. The results of Murphy suggested an excellent means of making a further analysis of the problem, since it would be of considerable aid if a species specificity, which apparently does not express itself normally until about hatching time, could be induced in an embryo still in the process of incubation. With this in mind a series of experiments was performed, the results of which were reported in abstract form (Sandstrom, '29 b), but are more fully described in this paper.

The problem was originally suggested by Dr. B. H. Willier, of The University of Chicago, and the work carried out at the Hull Zoölogical Laboratory under his direction. I am greatly indebted to him for his suggestions and criticisms.

MATERIAL AND METHODS

The method used in transplanting the tissue to the chorio-allantoic membrane of the chick was, except for minor variations in procedure, as described by Sandstrom ('32) and originally worked out by Willier ('24). The spleen was removed from a mature chicken and cut up in normal salt solution (38°C.) into pieces about 4 cu.mm. in size. The tissue was then implanted to the chorio-allantoic membrane of the host embryo on the eighth day of incubation. The egg was then sealed and returned to the incubator, and a second operation performed approximately twenty-four hours later, when a piece of duck kidney tissue about 1 to 4 cu.mm. in

size was implanted about a centimeter away from the spleen graft. The elapse of time between the two operations permitted the spleen tissue to become incorporated earlier than the duck tissue. After an additional period of incubation lasting eight days, both grafts were removed and fixed either in Bouin's or Zenker's solutions. They were sectioned in paraffin at 8μ , and stained either in iron-haematoxylin or eosin-methylene blue. The host embryos were fixed in Bouin's solution and preserved for future reference.

In all of the experiments the adult spleen tissue was secured from mature S. C. White Leghorn chickens. Embryos of S. C. White Leghorn chickens were used as hosts. White Pekin ducks were used as donors. They ranged in age from embryos of thirteen to twenty-eight days of incubation, ducklings of from twenty-nine to thirty-eight days, and adults.

The controls consisted of a similar series of transplants made without the presence of the spleen grafts.

DESCRIPTION OF RESULTS

The results of the transplantations of duck kidney on the chorio-allantoic membrane in conjunction with adult chicken spleen tissue do not differ in any appreciable way from those described in an earlier paper (Sandstrom, '32) which consisted of transplants of duck kidney tissue alone made on the chick membrane, and which are considered as the controls to this group of experiments. The results are tabulated in table 1. For convenience the transplants have been grouped into series according to the age of the donor tissue. The sizes of the adult chicken spleen grafts are included, and since these grafts often caused a distinct hypertrophy of the spleen of the host, the percentage of this increase over the size of the normally developed organ is also given. Reference should be made to the table in conjunction with the following description of the grafts.

TABLE 1

SERIES	CASE NO.	DUCK KIDNEY GRAFTS						ADULT CHICKEN SPLEEN GRAFTS			
		AGE OF HOST	Age of donor	Time on host	Total age of grafted tissue	Size of graft at the time of removal	Normal tissue	Necrotic tissue	Cellular reaction		
									Connective tissue	Lymphocytes	Granulocytes
I	61-11	Days	Days	Days	Days	Mm.					Size of graft at the time of removal
		9	13	8	21	3.70×2.80×3.73	+	—	—	—	Mm.
	61-18	9	13	8	21	5.50×5.00×2.70	+	—	—	—	6.00×0.61×1.05
	62-11	9	16	8	24	1.60×1.00×3.70	+	—	—	—	5.50×1.28×1.80
	62-12	9	16	8	24	2.05×1.80×1.33	+	—	—	—	None present
II	62-36	9	16	8	24	2.88×3.30×1.50	+	+	—	—	1.10×0.90×1.42
	63-3	9	19	8	27	4.00×2.60×3.53	+	+	+	+	2.10×1.40×3.26
	63-4	9	19	8	27	2.42×2.30×1.20	+	+	+	+	1.80×0.40×2.99
	63-5	9	19	8	27	1.80×0.70×2.50	+	+	+	+	0.70×0.80×0.79
	63-6	9	19	8	27	1.75×0.90×1.82	+	+	+	+	None present
III	63-12	9	19	8	27	1.00×0.50×0.60	+	+	+	+	2.60×3.80×4.41
	64-8	9	21	8	29	1.46×1.50×1.70	+	+	+	+	None present
	65-9	9	24	8	32	No tissue present	—	+	+	+	5.10×2.60×4.35
	65-10	9	24	8	32	1.81×1.10×1.30	—	+	+	+	4.90×1.30×3.87
	65-17	9	24	8	32	No tissue present	—	+	+	+	2.50×1.40×3.65
	65-18	9	24	8	32	No tissue present	—	—	+	+	3.70×2.60×4.10
	65-22	9	24	8	32	1.10×0.30×0.70	—	+	+	+	3.60×1.90×2.56
	65-27	9	24	8	32	0.81×0.20×1.50	+	+	+	+	3.60×1.50×3.54
	65-28	9	24	8	32	0.50×0.70×0.30	—	+	+	+	1.28×2.60×1.30
							—	+	+	+	2.30×0.80×2.32
											PERCENTAGE OF INCREASE IN SIZE OF HOST SPLEEN
											203
											304
											0
											0
											0
											252
											21
											0
											475
											0
											248
											533
											1042
											309
											306
											245
											114
											137

[illegible]

*Series I. Duck donors thirteen and sixteen days
of incubation*

The nephrogenous tissue taken from duck embryos thirteen and sixteen days of incubation grew and differentiated into apparently normal metanephric tissue while on the chorio-allantoic membrane of the chick host. The measurements show a considerable increase in the size of the graft over the pieces originally transplanted, and numerous mitotic figures are present in the cells of the differentiated tubules and the still undifferentiated nephrogenous tissue, indicating that the tissue was in an active growing condition at the time of removal. There is but one exception, case 62-36, in which a small area of necrotic tissue is present. There is no evidence that the host has resisted the transplant. Granulocytes are present in large numbers in the grafted tissue, but they are also present in large numbers in the normally developed kidney of the duck. There is no evidence of any lymphocytic infiltration, nor has any connective tissue invaded the transplant. In all respects, the grafts show conditions like those of the controls, even when the spleen grafts, which histologically show typical spleen tissue with numerous lymphocytes in the reticular structure, grew to a large size, or when the grafted spleen tissue caused a considerable hypertrophy of the spleen of the host as in cases 61-11 and 61-18. In the only case having necrotic tissue, the spleen graft showed an excellent growth, but caused no hypertrophy of the host's spleen. A comparison of the sizes of the grafts shows no essential differences from those of the control series.

Series II. Duck donors nineteen days of incubation

The histological details of the grafts resulting from the transplantation of kidney tissue from duck embryos that were nineteen days of incubation to the chick membrane in conjunction with adult chicken spleen is very much like that given in the description of the results of the control series

of equal age. Both normal and necrotic tissue is found in the same graft. In some it is present only to a slight degree, as, for example, cases 63-4 and 63-5, while in the remaining cases it is more pronounced. The cellular reaction varies. In case 63-3 some of the metanephric tissue seems normal, but has been split into two parts that are widely separated by a mass of connective tissue. This connective tissue occupies almost the entire graft, the actual measurements of the metanephric tissue being less than 1 cu.mm. Within the connective-tissue mass there are small aggregations of lymphocytes surrounding areas of necrosis. In the remaining grafts the connective-tissue infiltration into the grafted tissue is of the same general nature as the controls. The fibroblasts have worked into the center of the graft so as to more or less isolate groups of tubules. Small infiltrations of lymphocytes are present in one or two of the grafts, otherwise they do not form a conspicuous part of the histological detail. With but a single exception, case 63-3, as described above, the grafts of this series show no outstanding differences from those of the control series. The spleen grafts, however, were not very successful. Only in case 63-6 did the spleen tissue grow to any considerable size, while a decided hypertrophy of the spleen of the host was evident in this case, and also in case 63-3. The duck kidney tissue graft of 63-6, however, showed no sign of having been affected.

*Series III. Duck donors twenty-one to twenty-eight days
of incubation*

The histological detail of the kidney tissue grafts of this series is the same as that of the controls of comparable age. No growth of the tissue after transplantation is evident. At the time of removal, the grafted tissue was for the most part necrotic, although in some of the grafts a few of the tubules about the periphery appear to have been normal, and in the cells of these tubules occasional mitotic figures are found. Slight infiltrations of lymphocytes occurred in a few cases. Granulocytes, too, are present, but in lesser numbers than in

the previous series. The conditions present in this series of grafts differ in no respect from the parallel series of controls. This is true even though the spleen tissue grafts grew to a considerable size in many cases, and a hypertrophy of the host spleen effected in almost all cases.

*Series IV. Duck donors twenty-nine to thirty-eight days old
(one to ten days extrashell life)*

Grafts obtained from this series of transplants are consistently small and in most instances the kidney tissue necrotic. In a few of the grafts isolated tubules are to be found that still retain an apparently normal condition, for mitotic figures are found occasionally. The primary type of cellular reaction consisted of connective-tissue infiltrations into the grafted tissue. Lymphocytes and granulocytes are not very common. The grafts of adult chicken spleen tissue are for the most part small, but nevertheless seem to have affected the spleen of the host. In spite of this, there are no indications that the grafts of duck tissue differ in any way from the controls, either in size or in histological detail.

Series V. Adult duck donors

In neither of the two cases in this series were the grafts of adult chicken spleen tissue obtained nor was there any indication of a hypertrophy of the spleen of the host. In the grafts of duck kidney tissue, the tissue is completely necrotic. It is characteristically invaded and surrounded by connective tissue. These grafts are in no respect different from those of the control.

DISCUSSION

These experiments show that duck kidney tissue transplanted to the chorio-allantoic membrane of the chick is unaffected by the presence of adult chicken spleen grafts. Undifferentiated nephrogenous tissue from duck embryos grew and differentiated into apparently normal metanephric structures while on the chick membrane, while differentiated

metanephric tissue did not grow. Instead, it became necrotic and infiltrated with connective tissue. There are no appreciable differences between the results of these experiments and those reported by Sandstrom ('32) wherein duck kidney tissue alone was transplanted to the duck and chick. Since the capacity of duck kidney to grow and differentiate, and the nature and intensity of the reactions appearing against the grafts of differentiated tissue, were unaffected by the spleen transplants, the results are interpreted to mean that a precocious species specificity cannot be induced into the developing chick embryo by means of adult chicken spleen grafts.

The importance of the spleen in the resistance of the host to transplanted tissue, i.e., in specificity, has been the subject of considerable experimentation, but primarily by medical investigators interested in tumor growth. In general, there have been two methods of approach; either the tissue has been transplanted to splenectomized animals, or it has been transplanted in conjunction with additional spleen tissue. The results in both types of experiments have been conflicting. Oser and Pribram ('12) and Apolant ('13) obtained a lessened resistance to transplantable tumors in splenectomized mice and rats, whereas Bullock and Rohdenburg ('17, '18) failed to find that mouse tumor transplants were more successful in splenectomized rats than in normal rats. Jaffe ('31) calls attention to the fact that these discrepancies are probably due to the method of attack, and unless the remaining parts of the reticulo-endothelial system are completely blocked, the effect of splenectomy are compensated for by the proliferation of other parts of the system.

The transplantation of tumorous tissue in conjunction with spleen tissue also seems to give discrepant results which are not as readily explained. Murphy ('14, '26) transplanted embryonic and sarcomatous tissue of the rat to the chorio-allantoic membrane of the chick. He found that it grew well until the host had been incubated for eighteen days, after which time the transplanted tissue become necrotic and small

lymphocytes appeared in large numbers in, and around, the grafted tissue. This same manifestation of the development of specific properties in the host embryo was brought about at a much earlier stage in development when sarcomatous tissue of the rat was transplanted in conjunction with adult chicken spleen. Stevenson ('17 a, '17 b) transplanted sarcomatous tissue of the rat to the chorio-allantoic membrane of the chick in conjunction with adult chicken spleen, but failed to confirm the results of Murphy. His results are of interest, as he used the same type of sarcoma and varied the quantity of adult chicken spleen tissue. They are in accord with the results of the experiments reported in this paper. Also Bullock ('15) found that newborn rats, usually susceptible to mouse tumors, did not become more resistant when adult rat spleen tissue was grafted in conjunction with the mouse tumor tissue. However, it must be kept in mind that the transplants of normal duck kidney tissue may not be comparable to transplants of pathological tissue such as described above. In this respect Loeb ('21) emphasizes that transplantable tumors are unsuitable for an analysis of the individuality differential, since tumor tissue is capable of producing substances that hasten the reaction about the transplanted tissue.

The differentiated duck kidney tissue transplanted to the chorio-allantoic membrane of the chick in conjunction with adult chicken spleen tissue, as well as the transplants reported by Sandstrom ('32), became necrotic and infiltrated with connective tissue. Granulocytes, although present in the grafts, are not considered important in the reaction against the foreign tissue because they are abundant in the interstitial tissue of the normally developing kidney, decreasing in number as differentiation proceeds, but showing no significant increase in number in the grafts. Lymphocytes are also present, but rarely in such numbers to be considered an infiltration. This is quite in contrast to the results of Murphy ('14, '26), who found intense infiltrations of lymphocytes in the grafts of embryonic and sarcomatous tissue of the rat

after the chick host had reached the age of eighteen days of incubation, and at a much earlier stage when the rat tissue was grafted in conjunction with adult chicken spleen tissue. According to Loeb ('20), lymphocytes were rare in grafts of kidney tissue made between adults of various species of vertebrates. They were most common in grafts of guinea-pig kidney in the pigeon. He suggested that this might be due to the relatively greater number of lymphocytes normally present in the pigeon than in the other forms used. Stevenson ('17 a) found no significant change in the number of lymphocytes appearing in the grafts of sarcomatous tissue of the rat when transplanted to the chick in conjunction with spleen tissue. On the other hand, he did find an increase in the number of granulocytes.

The exact nature of the influence of the spleen tissue grafts on the host is unknown. When adult chicken spleen is grafted to the chorio-allantoic membrane of the chick, the grafted tissue, in some manner, causes the spleen of the host to hypertrophy. This has been shown by Murphy ('16, '26), Danchakoff ('16), and the experiments reported in this paper. Murphy believed that the spleen tissue grafts stimulated the spleen of the embryonic host to form small lymphocytes—a condition which does not occur in normal histogenesis until the late stages of embryonic development. Thus the intense infiltrations of small lymphocytes into the transplanted tissue as described by Murphy are accounted for. However, Danchakoff ('16), in a histological study of the hypertrophied spleen, failed to find such a stimulation toward the production of small lymphocytes, but found the increase in size was due to the intense production of granulocytes. An examination of a few of the enlarged spleens taken from hosts in this series of transplantations confirmed these observations. An increase of granulocytes would be more in accord with the results of Stevenson, as mentioned above.

SUMMARY

1. This paper is concerned with an attempt to induce a species specificity in the developing chick embryo, at an earlier age than it is presumed to develop, by means of adult chick spleen transplants.

2. Kidney tissue from duck embryos ranging from thirteen to twenty-eight days of age, from ducklings one to ten days after hatching, and from adult ducks was transplanted to the chorio-allantoic membrane of the chick in conjunction with adult chicken spleen tissue. The controls consisted of a similar series without the adult chicken spleen tissue grafts.

3. The grafts of adult chicken spleen tissue grew on the chorio-allantoic membrane of the chick, and histologically showed typical spleen tissue with numerous small lymphocytes in the reticular structure. In many cases the grafts of spleen tissue caused a decided hypertrophy of the host spleen.

4. The duck kidney tissue showed the same capacity to grow in the presence of spleen tissue grafts, and enlarged spleens of the host, as it did in the controls. Undifferentiated metanephrogenous tissue grew and differentiated to apparently normal metanephric structures. Differentiated metanephric tissue, however, did not grow, but became necrotic and invaded with connective tissue. Lymphocytes and granulocytes took no more active part in the resistance of the host in these experiments than they did in the controls.

5. The results of these transplantations are interpreted to mean that adult chicken spleen tissue is not capable of inducing a precocious species specificity in the developing chick embryo.

6. Casual examination of some of the hypertrophied spleens of host embryos indicates that a stimulation in the production of granulocytes, and not of lymphocytes, is the cause of the increase in size of this organ.

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ON CHANGES IN THE SIZE, SHAPE, AND DISTRIBUTION OF THE BASOPHIL SUBSTANCE IN THE NEUROCYTOSOME AFTER FIXATION IN DIFFERENT FLUIDS¹

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ONE PLATE (FIFTY-TWO FIGURES)

INTRODUCTION AND HISTORY

The importance of the tigroid substance in nerve cells, which has been pointed out by Nissl ('89), stimulated numerous investigations relative to its nature.

That this substance, which has been variously termed, but is popularly known as Nissl's granules, has no physical counterpart in the living cell has been shown by Mott ('12) and Marinesco ('12). The latter made observations on living spinal and sympathetic ganglion cells with the aid of the ultramicroscope and direct illumination under high magnification. In summarizing his findings he stated that:

. . . . weder die Nissl'schen Urkörperchen, noch die Neurofibrillen, noch das Karyoplasma den physicalischen Character haben, welchen die durch verschiedenen histologischen Verfahren entstandende Structur anzeigen. Diese Untersuchungen haben uns gezeigt, dass das Protoplasma ein Kolloidkomplex ist, dessen Structur von einer Zellart zur anderen wechselt und der durch die verschiedenen Fixiermittel, die auf die kolloiden Granulationen, die Nervenfasern und das Karyoplasma eine fällende Wirkung ausüben, verändert wird.

The living protoplasm, he concluded, may be said to behave like a negative colloid which is 'homogenized' by alkali and precipitated by acids.

¹ Contribution no. 169.

Hopkins ('24) studied the effects of different fixatives on the Nissl granules in the anterior gray-column cells of the spinal cord of the white rat. He used Zenker's fluid, various grades of alcohol, and formalin, all without and with the addition of varying amounts of acetic acid. He found that the addition of acetic acid favorably influenced the appearance and size of Nissl granules; furthermore, different fixation of the same type of cell (from the same tissue and animal) may reveal either diffused basophilic substance throughout the greater portion of the cytoplasm or definitely formed tigroid bodies. Hopkins concluded that "The appearance of Nissl substance in fixed preparations of nerve cells is largely dependent upon the substances used for fixation."

The development of Nissl granules in the embryo was studied by Scott ('99), Van Biervliet ('00), and others. Scott ('99) followed the development of Nissl substance in the anterior gray-column cells of the spinal cord. He found that at a very early embryonic stage these cells consisted almost entirely of a nucleus rich in stainable matter. Later on, as the cells increased in size, their nuclei became less rich in chromatin and a 'cap' of peculiar nature, stainable with toluidin blue, appeared close to the nucleus. Still later, the substance of the 'cap' became uniformly distributed throughout the cell and finally assumed the form of granules, which formed the 'spindles' found in the adult cells. This progressive course of development led him to believe that these spindles were derived from the nuclei of the nerve cells.

According to Van Biervliet ('00), Zolovtsoff of Moscow studied the development of Nissl granules in human embryos. Zolovtsoff reported that the course of development of Nissl granules is from the perinuclear zone toward the periphery of the cell body.

Contrary to Zolovtsoff, Van Biervliet ('00) after a similar study expressed the opinion that Nissl granules appeared first near the cell periphery and then spread centrally toward the nucleus, and that they were formed from the homogeneous basophilic 'storehouse' in the cytoplasm. Nicholson ('23, '24)

stated that in the regeneration of nerve cells after axon injury the iron-containing Nissl granules appeared first at the periphery of the nucleus and then spread to the whole cytosome.

Some conception of the chemistry of Nissl granules may be gathered from the researches of Macallum ('98), Nicholson ('23, '24), Mühlman ('29), and others. Macallum ('98) found that these granules resisted peptic digestion, were slowly digested by trypsin, and contained some phosphorus and iron. He concluded that Nissl substance is of the nature of a nucleoprotein and contained both iron and phosphorus.

Nicholson ('23, '24), after unilateral ligation or tearing of the hypoglossal nerve in the adult white rat, and using the same preparations for both the Nissl-granule technique and microchemical test for iron, obtained identical pictures in both cases. He concluded that the Nissl substance is or contains an iron-holding protein.

According to Mühlman ('29), in young embryos (rabbit, cat, guinea-pig, and man) nuclein and globulin were present in Nissl granules, while during the process of growth the amount of nuclein increased at the expense of globulin and as a result of this change no globulin was found in the granules of the adult.

The present communication deals with a study of the effects produced by fixation with the salts of various heavy metals on the morphology and distribution of Nissl granules. The cells chosen for study were the anterior gray-column cells of the spinal cord and the Purkinje cells of the cerebellum.

MATERIALS AND METHODS

Samples from the spinal cord and cerebellum of the dog, cat, and rabbit were fixed for about twenty-four hours in 0.5M solutions of the following salts:

AlCl_3 , $\text{Al}_2(\text{SO}_4)_3$, CdCl_2 , $\text{Cr}(\text{C}_2\text{H}_3\text{O}_2)_3$, $\text{Cr}(\text{NO}_3)_3$, $^*\text{Cr}_2(\text{SO}_4)_3$, $^*\text{Cu}(\text{C}_2\text{H}_3\text{O}_2)_2$, CuCl_2 , FeCl_3 , $\text{Fe}(\text{NO}_3)_3$, $\text{Fe}_2(\text{SO}_4)_3$, $\text{Hg}(\text{C}_2\text{H}_3\text{O}_2)_2$, $^*\text{HgCl}_2$, $\text{Hg}(\text{NO}_3)_2$, HgSO_4 , SnCl_4 , $\text{Sn}(\text{SO}_4)_2$, $\text{UO}_2(\text{NH}_4)_2\text{Cl}_4$, $\text{UO}_2(\text{NO}_3)_2$, $\text{Zn}(\text{C}_2\text{H}_3\text{O}_2)_2$, ZnCl_2 , $\text{Zn}(\text{NO}_3)_2$, and Zenker's fluid with 5 per cent acetic acid.

* Saturated before 0.5M concentration was reached.

The acid corresponding to the anion was added in some instance to keep the salt in solution. Seven per cent of sulphuric acid was required for HgSO_4 and not over 2 per cent for the others. Another group of samples from the spinal cord, cerebellum, and dorsal-root ganglia of a cat was fixed in each of four saturated solutions of HgCl_2 , the pH of which was adjusted to 1, 2, 3, and 5.5, respectively. The approximate pH value of all the salt solutions was determined with the aid of indicators and a color chart taken from Clark ('28).

The tissues were embedded in paraffin, cut into 10μ sections, and stained with a 1 per cent aqueous solution of thionin blue.

OBSERVATIONS

1. The basophil substance of the cytosome presented a variable picture and appeared in the form of: Deeply stained and clearly defined masses, which were coarse (fig. 18), medium (fig. 44), or fine (fig. 13); faintly stained masses, with an apparently indefinite outline of coarse (fig. 35), medium (fig. 26), or fine (fig. 24) dimensions; irregular or elongated granules with branching processes which anastomosed and gave the basophil substance the appearance of a coarse (fig. 28) or fine (figs. 1, 3, 10, and 11) network; spheroid bodies of varying size (figs. 40 and 4); an apparently homogeneous basophil coloration of the cytoplasm which was limited either to a part (figs. 33 and 43) or distributed throughout the cytosome (figs. 34 and 42).

2. The cytosome usually included more than one type of granule.

3. In the anterior gray-column cells the basophil granulations (regardless of size, shape, or density of granules and fixative employed) had a similar distribution within the cell body. This was not the case with the Purkinje cells.

4. It became apparent from the evaluation of the individual salts as Nissl-granule fixatives (considering the number, size, and distinctness of these granules as criteria) that on the whole the chlorides gave the best results, while sulphates, nitrates, and acetates followed in the order named. From

the standpoint of the metal employed it appeared that zinc, cadmium, mercury, and tin were superior to copper, iron, uranium, and aluminum in the order named.

5. The pH of the fixing fluid within certain limits on the acid side was not the determining factor in the appearance and morphology of the basophil substance (table 1).

TABLE 1

A list of the approximate pH values of the single salts employed

SALT	pH	FIGURES	SALT	pH	FIGURES
AlCl ₃	3	1, 2, 3	Hg(C ₂ H ₃ O ₂) ₂	3.7	28
Al ₂ (SO ₄) ₃	1.2	4, 5, 6	HgCl ₂	2.9	29, 30, 31
CdCl ₂	5.6	7, 8, 9	Hg(NO ₃) ₂	1.2	32, 33, 34
Cr(C ₂ H ₃ O ₂) ₃	5.8	10, 11, 12	HgSO ₄	1.2	35, 36, 37
Cr ₂ (SO ₄) ₃	3.6	16, 17	SnCl ₄	1.2	38, 39
Cr(NO ₃) ₃	5	13, 14, 15	Sn(SO ₄) ₂	¹	40
Cu(C ₂ H ₃ O ₂) ₂	5.3	16, 17	UO ₂ (NH ₄) ₂ Cl ₄	1.5	41
CuCl ₂	4.4	18	UO ₂ (NO ₃) ₂	2.2	42, 43
FeCl ₃	1.8	19, 20	Zn(C ₂ H ₃ O ₂) ₂	5.5	44, 45, 46
Fe(NO ₃) ₃	1.8	21, 22, 23	ZnCl ₂	2	47, 48, 49
Fe ₂ (SO ₄) ₃	2	24, 25	Zn(NO ₃) ₂	4.5	50

¹ Hydrolyzed.

DISCUSSION

1. Nissl granules

The morphology of individual granules varies greatly with the type of fixative used, the plane and thickness of the section, and the degree of staining intensity.

Plate 1 illustrates the influences exerted by the several fixing fluids. The differences caused by varying the plane of section of a cell are well known and are probably best demonstrated by primary motor neurons when some are cut parallel and others perpendicular to the long axis of the granules. Experience has proved what should have been plain to certain workers—that the thicker the section, the more apparently numerous are the granules. Variations in the staining intensity likewise influence the sharp delineation (and hence the apparent size) of granules.

2. *Nissl pattern*

It may be assumed from the figures in plate 1 that the picture of the basophil substance depended upon the preliminary treatment (fixation) of the tissue. A more detailed study of the Purkinje cells revealed that this substance appeared in some cases as a homogeneous field occupying either a part (fig. 12) or the entire cytosome (figs. 34 and 37), while in other cases it appeared in the form of granules that varied in number, size, and distribution. This is in agreement with the findings of Hopkins ('24). When attention was directed to the anterior gray-column cells, it became apparent that the only morphological difference between their basophil substance and that of the Purkinje cells was in the constant pattern of distribution. This difference leads to the assumption that the Nissl pattern may be variable in one type of cell (Purkinje) and constant in another (cord), even when identically treated.

What causes this difference in pattern? Does the Nissl substance arrange itself at random or does it follow some predetermined pattern? As for the Purkinje cells, this substance was virtually without order in all the preparations. In the cells of the cord, on the other hand, it was always distributed throughout the entire cell body, which indicates that some factor (absent in the case of the Purkinje cells) was probably influencing a uniform distribution in this case. That the factor preexisted is evidenced by the fact that identical technical steps were applied to both types of cells. The importance of determining the preexistence of a factor that governs the ultimate distribution of Nissl granules in fixed preparations becomes evident when one considers its relation to such work as typing of cells based on the arrangement (distribution) of these granules reported by Warrington and Griffith ('04), Carpenter and Conel ('14), Clark ('26), Sheinin ('30), and others.

For, if it is true for all types of nerve cells that the Nissl pattern may be as variable as are the size and shape (and occurrence?) of granules, then the value of this mode of cell

typing and the conclusions drawn therefrom have been considerably exaggerated. Some information on the possible preexistence of a pattern-determining factor may be gained from the following summarizations:

Fischer ('94, '95, '99) produced many types of granules by precipitating protein solutions with standard fixatives. He also found that not all protein solutions are precipitable by all fixatives. Sheinin and Davenport ('31) reported that some heavy-metal salts precipitated albumin, but not gelatin. Hardy ('99 a, '99 b, '00) stated that whether coagulation or precipitation of a protein occurred depended upon the concentration of the protein, whether the iso-electric point was reached quickly or slowly, and the presence or absence of mechanical agitation.

Since Nissl granules have not been observed in the living nerve cell and since the cytoplasm is generally assumed to behave as a protein solution, it is inferred that laws governing coagulation and precipitation of protein solutions may be applicable to nerve cells.

The investigations of the workers already mentioned may probably explain the variations next to be described.

A. Purkinje cells. *a.* Where no granules were apparent. The possibilities are that either the fixing fluids used were of the non-precipitating type relative to the nerve cell proteins,² or that the protein solution in these cells was too dilute to produce a precipitate. But since the same fluid produced granulations in the anterior gray-column cells, the failure of the appearance of granules in these cells must have been due to the low concentration of proteins in their cytoplasm.

b. Where granules were apparent. In such instances it must be assumed that the stainable proteins in the cytoplasm

² Both in the Purkinje cells and anterior gray-column cells the possibility of the influence of mechanical agitation must be discarded, since no agitation was used. The idea of unlike proteins in each type of cell must likewise be abandoned, since there are no data at hand indicating such a difference. Similarly, there is no reason to assume that the pH of different types of nerve cells is at variance due to the differences in type or concentrations of the proteins.

were in sufficient concentration to be precipitated and that the random arrangement of the granules was due to a similar preexisting arrangement of the proteins. This leads one to believe that the preexisting factor in these cases was the non-homogeneous concentration of the protein which was inconstant in distribution and which resulted in the inconstant (variable) Nissl pattern.

B. Anterior gray-column cells. *a.* Where no granules were apparent. Since there were no such instances observed, it is possible that the concentration of stainable proteins in these cells was greater than in Purkinje cells.

b. Where granules were apparent. The constant presence of basophilic material and its constant uniform distribution throughout the cytoplasm indicate that in these cells the preexisting factor is the homogeneous concentration and constant distribution pattern of the proteins in the cytoplasm which resulted in a constant Nissl pattern.

From what has been said it is fairly reasonable to infer that in general the appearance of the basophil substance depends upon the type of fixative employed and the protein concentration. The Nissl pattern depends upon the constancy or inconstancy of the preexisting distribution pattern of stainable proteins in the cytoplasm.

3. The efficacy of the cation or anion of the fixing fluid as a Nissl-granule producer

The findings cited under "Observations" indicate that no definite conclusions can be drawn, though some have proved to be superior to others. It appears that the nature of the salt molecule as a whole is as great a factor as is the cation or anion.

4. Influence of the pH of the fixing fluid on the appearance of the granules

Zircle ('27, '28) studied the effect of the hydrogen-ion concentration upon fixation. In his opinion the pH of the fixative greatly influenced the final image produced, for certain

cell constituents appeared at one pH, while others first became demonstrable at another. He stated that if the fixative is too acid it will fix the chromatin, but not the mitochondria; if it is too basic it will fix the mitochondria, but not the chromatin.

The present investigation shows that varying the hydrogen-ion concentration on the acid side within pH limits of 1.2 and 5.8 does not have any determining effect on the appearance of the Nissl substance. Good or fair granules were obtained both at a pH of 5.5 with $\text{Zn}(\text{C}_2\text{H}_3\text{O}_2)_2$ (figs. 44, 45, and 46) and at a pH of 1.2 with $\text{Al}_2(\text{SO}_4)_3$ (figs. 4, 5, and 6). On the other hand, poor granules were obtained both at a pH of 5.8 with $\text{Cr}(\text{C}_2\text{H}_3\text{O}_2)_3$ (figs. 10, 11, and 12) and at a pH of 2.2 with $\text{UO}_2(\text{NO}_3)_2$ (figs. 42 and 43). In case of the HgCl_2 series with the varying hydrogen-ion concentration it was found that a similar basophil picture was present at a pH of 2, 3, and 5.5, while at a pH of 1 the granules were not apparent. This indicates that at a pH between 2 and 5.5 the hydrogen-ion concentration had no determining effect on the results, while it did have at a pH of 1. These findings may be said to agree with those of Hopkins ('24), who found that acetic acid, when added to a fixative in moderate amounts (up to 20 per cent), greatly improved the appearance of Nissl granules, but when an excess of acid was added the reverse was true.

CONCLUSIONS

The morphology (and occurrence?) of Nissl granules depends upon the fixative used, the plane and thickness of the section, and the intensity of staining.

The distribution of these granules, which may be constant or inconstant in different types of cells, is governed by a pre-existing factor. This is probably expressed in the mode of concentration and distribution of stainable basophil proteins in the living cell.

The typing of cells, based on the arrangement of their Nissl material, is probably justifiable only when an essentially similar pattern is obtained after various fixing methods.

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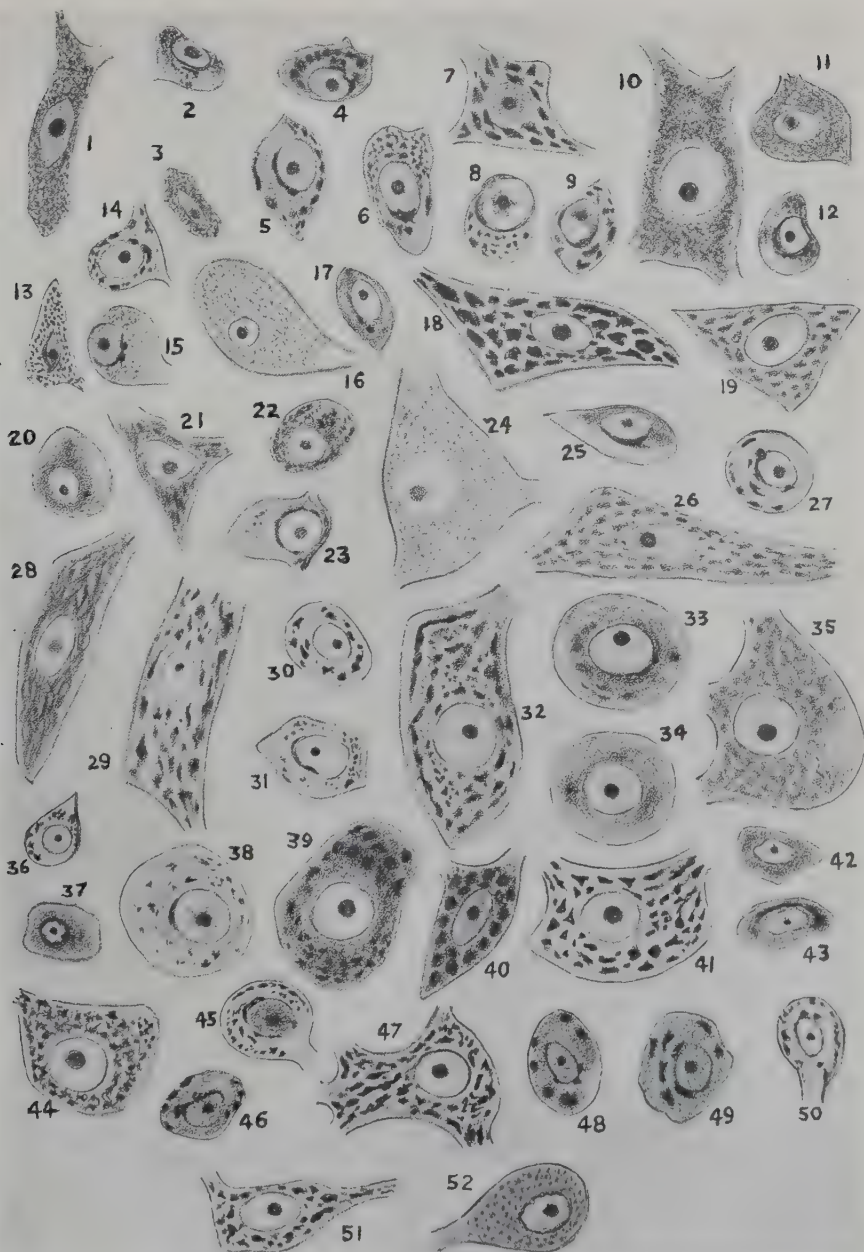
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PLATE 1

EXPLANATION OF FIGURES

All drawings were made with a Spencer 2-mm. apochromatic oil-immersion objective and a Leitz 6 $\times$ periplane ocular. Camera-lucida outlines were first made and the Nissl granules then filled in. The figures represent cells at a magnification of 567 diameters taken from sections cut 10 μ in thickness. "Cord" denotes a cell from the ventral gray column. "Cerebellum" denotes a Purkinje cell.

- 1 (cord), 2 and 3 (cerebellum) Fixed in AlCl_3 .
- 4, 5, and 6 (cerebellum) Fixed in $\text{Al}_2(\text{SO}_4)_3$.
- 7 (cord), 8 and 9 (cerebellum) Fixed in CdCl_2 .
- 10 (cord), 11 and 12 (cerebellum) Fixed in $\text{Cr}(\text{C}_2\text{H}_3\text{O}_2)_3$.
- 13 (cord), 14 and 15 (cerebellum) Fixed in $\text{Cr}(\text{NO}_3)_3$.
- 16 (cord), 17 (cerebellum) Fixed in $\text{Cr}_2(\text{SO}_4)_3$.
- 18 (cord) Fixed in CuCl_2 .
- 19 (cord), 20 (cerebellum) Fixed in $\text{Cu}(\text{C}_2\text{H}_3\text{O}_2)_2$.
- 21 (cord), 22 and 23 (cerebellum) Fixed in FeCl_3 .
- 24 (cord), 25 (cerebellum) Fixed in $\text{Fe}(\text{NO}_3)_3$.
- 26 (cord), 27 (cerebellum) Fixed in $\text{Fe}_2(\text{SO}_4)_3$.
- 28 (cord) Fixed in $\text{Hg}(\text{C}_2\text{H}_3\text{O}_2)_2$.
- 29 (cord), 30 and 31 (cerebellum) Fixed in HgCl_2 .
- 32 (cord), 33 and 34 (cerebellum) Fixed in $\text{Hg}(\text{NO}_3)_2$.
- 35 (cord), 36 and 37 (cerebellum) Fixed in HgSO_4 .
- 38 and 39 (cerebellum) Fixed in SnCl_4 .
- 40 (cord) Fixed in $\text{Sn}(\text{SO}_4)_2$.
- 41 (cord) Fixed in $\text{UO}_2(\text{NH}_4)_3\text{Cl}_4$.
- 42 and 43 (cerebellum) Fixed in $\text{UO}_2(\text{NO}_3)_2$.
- 44 (cord), 45 and 46 (cerebellum) Fixed in $\text{Zn}(\text{C}_2\text{H}_3\text{O}_2)_2$.
- 47 (cord), 48 and 49 (cerebellum) Fixed in ZnCl_2 .
- 50 (cerebellum) Fixed in $\text{Zn}(\text{NO}_3)_2$.
- 51 (cord), 52 (cerebellum) Fixed in Zenker's fluid plus 5 per cent acetic acid as a control.



A SECOND BRIEF NOTE ON 'FLOATING CLAVICLE'

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In *The Anatomical Record* for 1919 (vol. 16, pp. 379-380) I drew attention to a condition which I termed 'floating clavicle,' which, as far as I knew, had not previously been described. In this condition, the inner end of the clavicle, instead of constantly impinging upon and forming a joint with its fulcrum in the articular facet on the clavicle, lay quite free and could be moved backward, forward, inward, and outward. The range of its mobility was limited by its capsule instead of by bony obstruction as in normal subjects.

When I wrote the above article, I had seen three such cases, all of which were unilateral, though it is probable that I might have discovered several more had I made a special point of looking for them. Since then I have seen a dozen such cases, one of which was bilateral, all the remainder being unilateral and nearly all on the left side.

The bilateral case occurred in a healthy young female, aged thirty-two years, and it caused her no inconvenience whatever; in fact, the patient was totally unaware that her clavicles were in any way unusual. When she moved her arms backward, the clavicles separated, leaving a space of about $1\frac{1}{4}$ inches between their respective sternal ends. On bringing the arms forward until the hands met, the clavicles almost impinged upon one another. The only abnormality that I could detect was that the clavicular insertion of the two sternomastoids was almost absent, the sternal insertion being correspondingly larger. The patient's muscular development was, on the whole, above the average.

